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Crystallography at 100

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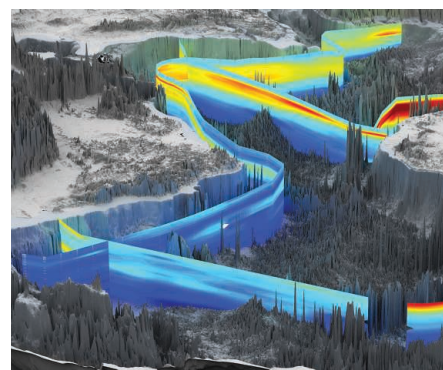
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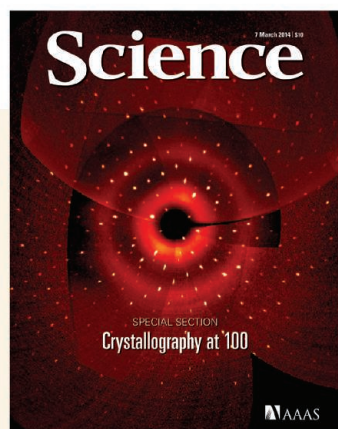
ON THE WEB THIS WEEK

>> Science Podcast

Listen to stories on 100 years of crystallography, firming up the link between climate change and malaria, and more.

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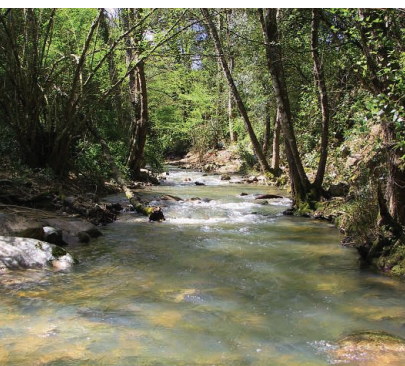
COVER

Precession image reconstructed from x-ray diffraction data collected from a benzene single crystal. For the past 100 years, x-ray crystallography has been a key tool for determining the structures of ever more complex chemical and biochemical systems. See the special section beginning on page 1091 and at www.sciencemag.org/special/crystallography.

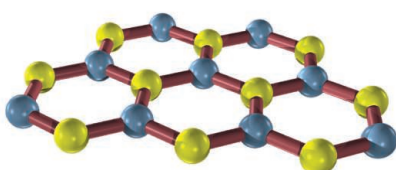
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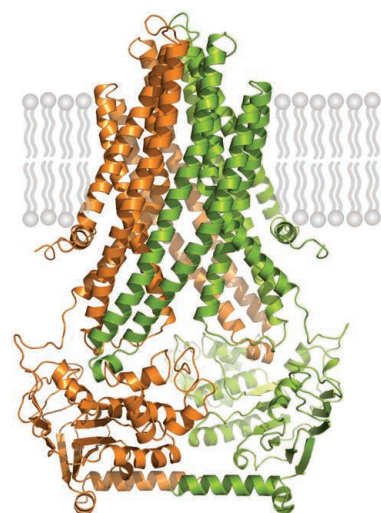
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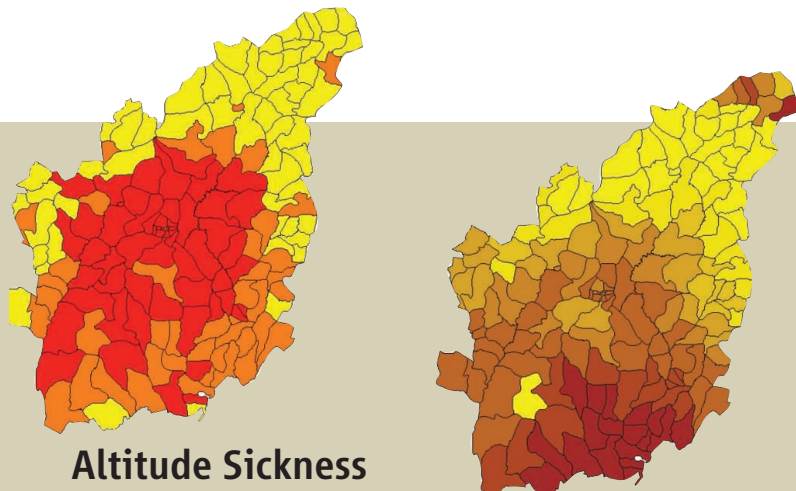
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Altitude Sickness

Whether the incidence of malaria will be (or has been) affected by the warming climate is poorly resolved. Global-scale analyses are fraught with technical difficulties, including problems with separating out changes resulting from destruction of mosquito habitat, insecticide use, and antimalarial drug use from multidecadal trends in climate change. **Siraj *et al.*** (p. 1154) performed parallel analyses of highland malaria in Ethiopia and Colombia to ask whether interannual changes in temperature could explain variation in malaria incidence with altitude. Modeling the data confirmed that malaria moves up in elevation in warmer years and allowed estimates of how many more millions of cases could be expected in tropical highland areas if the mean temperature increased by 1° to 3°C.

A Soft Molecular Spring

In noncontact atomic force microscopy, extremely high resolution has been achieved by attaching a terminal CO molecule to the metal scanning probe tip. This CO molecule can undergo torsional vibrations, and a full understanding of the imaging mechanism requires a measure of this spring constant. **Weymouth *et al.*** (p. 1120, published online 6 February; see the Perspective by **Salmeron**) used lateral force microscopy, in which the tip vibrates laterally across the surface, to determine this torsional constant. The stiffness of the isolated CO molecules on the tip was much less than that for CO molecules adsorbed on planar surfaces.

Nanoimaged Polaritons

Engineered heterostructures consisting of thin, weakly bound layers can exhibit many attractive electronic properties. **Dai *et al.*** (p. 1125) used infrared nanoimaging on the surface of hexagonal boron nitride crystals to detect phonon polaritons, collective modes that originate in the coupling of photons to optical phonons. The findings reveal the dependence of the polariton wavelength and dispersion on the thickness of the material down to just a few atomic layers.

Limited Stability

Deep ocean circulation is thought to be stable during warm, interglacial periods. **Galaasen *et al.*** (p. 1129, published online 20 February) constructed a highly resolved record of North Atlantic Deep Water production during the last

interglacial period, around 128,000 to 116,000 years ago. The findings reveal large, centennial-scale reductions—in contrast to the prevailing paradigm. These changes occurred in an ocean warmer than that of today, but in a temperature regime similar to that expected because of global warming, raising the possibility that future ocean circulation, regional climate, and CO₂ sequestration pathways could be impacted.

Crossing the Membrane

Adenosine triphosphate (ATP)-binding cassette (ABC) transporters couple ATP hydrolysis to the translocation of a wide variety of substrates across cell membranes. **Srinivasan *et al.*** (p. 1137) describe the structure of a yeast mitochondrial transporter involved in Fe-S protein biogenesis. The structure reveals bound glutathione, which suggests that glutathione is part of the translocated substrate. **J. Y. Lee *et al.*** (p. 1133) describe the structure of a bacterial ABC transporter that confers protection against silver and mercury. This protein also binds glutathione derivatives. The structure provides insight into how ligand interactions are coupled to ATP hydrolysis.

Evolutionary Duality

When ecological divergence involves traits that also contribute to mating behavior, such divergence could lead to rapid reproductive isolation and speciation. However, there are very few experimental demonstrations of such “dual” traits. **Chung *et al.*** (p. 1148, published online 13 February) now demonstrate that specific cuticular hydrocarbons are a dual trait that affects both

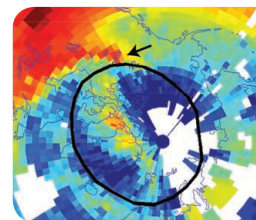
desiccation resistance and mate choice in the widely distributed Australian species *Drosophila serrata*. These compounds have largely been lost from its rainforest-restricted, desiccation-sensitive, closely related sibling *D. birchii*.

Carbapenems Through the Looking Glass

The carbapenem class of antibiotics is a critical weapon in the ongoing fight against drug-resistant bacteria. Microbial biosynthesis of these compounds, which contain a strained β -lactam ring motif, proceeds via a precursor that has the wrong configuration at one of the ring carbons. **Chang *et al.*** (p. 1140) combined x-ray crystallography with multiple spectroscopic probes to map out the mechanism by which the CarC enzyme inverts the precursor configuration to its mirror image.

Persistent Plume

Energy from the solar wind is carried into Earth's magnetosphere and ionosphere through the reconnection of magnetic lines. When this coupling is strong, changes in the solar wind cause geomagnetic storms that perturb the magnetosphere and allow a plume of cool plasma to be released outward. As the plume rises from the ionosphere, it extends the boundary of its plasmasphere origin toward the magnetopause. Using simultaneous ground-based and satellite observations, **Walsh *et al.*** (p. 1122; see the Perspective by **Borovsky**) show that this plume can persist for hours, extending all the way from the ionosphere to the magnetopause.



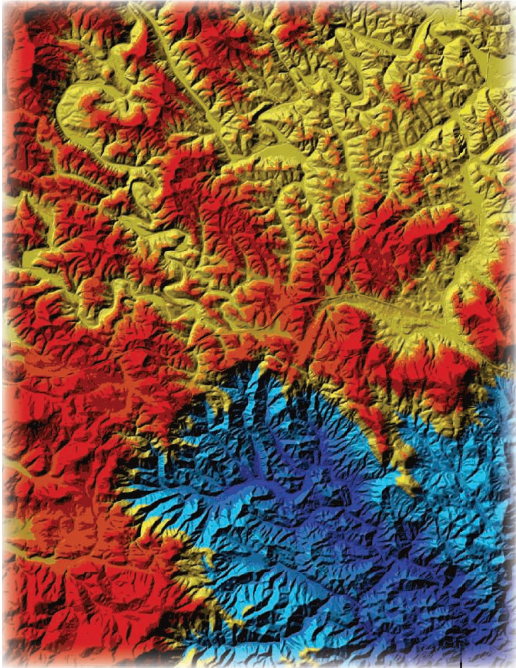
Keeping HIV at Bay?

Preexposure prophylaxis involving daily doses of drugs can, with variable success rates, interrupt HIV transmission for individuals at high risk of acquiring HIV infection. However, one reason for the variability seen in the response to such drugs is a lack of adherence to the prescribed regimen. **Andrews *et al.*** (p. 1151) formulated a potent integrase inhibitor as a long-acting agent that protected macaques from repeated intrarectal challenges of simian HIV. Decay of plasma levels of drug were associated with increased susceptibility to infection after virus exposure. The drug levels required for a high degree of protection could potentially be achieved with quarterly injections in humans.

Additional summaries

River Reorganization

As rivers flow, they slowly transform the landscape. Their channels migrate, banks erode, and rivers can even flow backward if



merged with another river. To understand how river basins balance erosional forces with regional tectonic uplift, **Willett *et al.*** (p. 1117) analyzed maps of a proxy for river elevation

and horizontal movement of river drainage divides across three large river systems in China, Taiwan, and the United States. Along with numerical modeling, the results demonstrate the degree to which these basins are at topographic equilibrium. The changing connectivity and topography of river networks influences how species migrate and how much material is delivered to larger bodies of water.

Immune Variation

It is difficult to determine the mechanistic consequences of context-dependent genetic variants, some of which may be related to disease (see the Perspective by **Gregersen**). Two studies now report on the effects of stimulating immunological monocytes and dendritic cells with proteins that can elicit a response to bacterial or viral infection and assess the functional links between genetic variants and profiles of gene expression. **M. N. Lee *et al.*** (p. 1119) analyzed the expression of more than 400 genes, in dendritic cells from 30 healthy subjects, which revealed how expression quantitative trait loci (eQTLs) affect gene expression within the interferon- β and the Toll-like receptor

3 and 4 pathways. **Fairfax *et al.*** (p. 1118) performed a genome-wide analysis to show that many eQTLs affected monocyte gene expression in a stimulus- or time-specific manner.

Plant Epigenetics

Quantitative trait loci (QTLs) are genetic regions associated with phenotypic traits that help to determine the underlying genetics controlling the magnitude of a specific trait. **Cortijo *et al.*** (p. 1145, published online 6 February; see the Perspective by **Schmitz**) identified epigenetic QTLs associated with differences in methylation marks (epiQTLs) controlling flowering time and root length in the model plant *Arabidopsis*. These epiQTLs were mapped in genetically identical lines that differ only in their methylation marks. A small number of QTLs were able to explain up to 90% of the heritable variation in these traits. Thus, in plants, the heritability of some complex traits can be determined by epigenetic variation.



Gautam R. Desiraju is a professor of chemistry at the Indian Institute of Science, Bangalore, India, and president of the International Union of Crystallography. E-mail: desiraju@sscu.iisc.ernet.in

Crystallography and Geopolitics

DEVELOPED AND DEVELOPING NATIONS RECOGNIZE THAT INNOVATION IS KEY TO THEIR ECONOMIES. Connecting this with the discipline of crystallography may not seem immediately apparent, but during the past century, understanding the structure of matter has transformed industries and created new frontiers, from the design of new medicines and materials to assessing the mineral content of Mars. The future global economy will be determined by progress in cutting-edge fields. However, the playing field is not level in crystallography, which is why the International Union of Crystallography (IUCr) and the United Nations Educational, Scientific and Cultural Organization (UNESCO) have marked 2014 as the International Year of Crystallography. The aim is to improve public awareness of the field, boost access to instrumentation and high-level research, nurture “home-grown” crystallographers in developing nations, and increase international collaborations for the benefit of future generations.

The development of scientifically influential ideas is most prominent in wealthy countries. Those nations should continue to invest in science to remain economically advanced. They should not try to live off their existing scientific capital and hope to compensate for future shortfalls through business, management, and outsourcing to ostensibly “cheaper” countries. A developing country, on the other hand, needs to invest in science to define its own technologies and find a voice in international forums. But any country, wealthy or not, that lacks a healthy native scientific enterprise cannot make up the deficit by importing science from more scientifically advanced nations. Such attempts can never lead to a stable scientific culture or society. Embracing the relevance of science in one’s life and growing science locally are the true measure of a country’s scientific success, not the number of Nobel Prizes that have been given to people who were born, lived, or worked in that country.

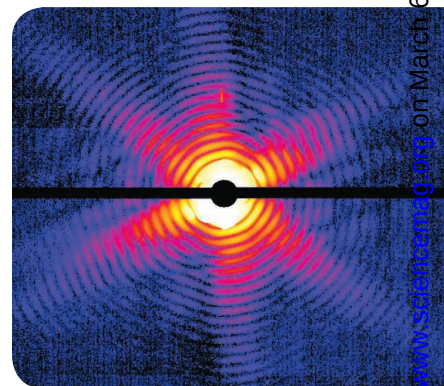
The newly advancing economies of Brazil, Russia, India, China, and South Africa (the so-called BRICS nations) are investing heavily in science and technology. As a result, crystallography’s future may well lie in these parts of the world, which have people power and increasing economic muscle. By 2030, China, India, and the African continent will have 1.5 billion people each, most of whom will be educated. All of the Western world will by then have just 1 billion people. This means that “Chindiafrica,” with its 4.5 billion people, could exert a substantial geopolitical and scientific influence in the world, with the focal point being the Indian Ocean rather than the northern Atlantic.

The International Year of Crystallography has placed a special focus on Africa, Latin America, and Asia. The efforts include a plan for “open laboratories” that, in partnership with industry, will enable students in far-flung lands to have hands-on training in modern techniques and expose them to cutting-edge research in the field. Open labs in Uruguay, Ivory Coast, and Algeria are already on the anvil. The IUCr also is running a training program in crystallography, in which students from sub-Saharan Africa can obtain a Ph.D. in the field in more advanced locales, such as the Universities of the Witwatersrand and Cape Town in South Africa.

More-powerful synchrotrons and free-electron laser facilities will be needed to determine increasingly complex structures. IUCr and UNESCO hope that setting up such facilities will assist in expanding and strengthening crystallography beyond 2014. A good example of this is in Jordan, where governments are working together to construct the Synchrotron-light for Experimental Science and Applications in the Middle East (SESAME). Brazil has impressive synchrotron facilities where collaboration among scientists from other Latin American countries is encouraged. More forums to guide research priorities, multinational partnerships, and funding arrangements are needed. What is most important is for scientists to interact seamlessly with the enormous amounts of data that will be generated in crystallography so that anyone, anywhere, can get any kind of structural information and use it profitably. Crystallography is a facilitating discipline, and this is why it will always endure.

– Gautam R. Desiraju

10.1126/science.1252187



VIROLOGY

Opening Pandora's Box

The first woman on Earth, Pandora, had a "box," or rather a jar, that Zeus commanded her to safeguard and never open. Of course she opened it, and thus evil spread around the world. Recently, an extraordinarily distinctive group of giant viruses that parasitize amoebas were described and named Pandoravirus, not because they contain all evil but merely because they are jar-shaped. Le gendre *et al.* have added to this still-tiny pantheon with another jar-shaped viral particle 1.5 μm long, containing a rather diminutive 600-kb AT-rich genome (as compared to the up to 2.8-Mb genome seen in Pandoraviruses) and a cytoplasmic replication machinery resembling that of the original Megaviridae. The authors named the virus Pithovirus because Pandora's jar was called a "pithos" in ancient Greek. This virus was revived from a Siberian permafrost sample and infects amoebas. Although named for the jar and not its contents, given its origins, this discovery hints that viruses more evil than Pithovirus might be revived as the tundra melts. — CA

Proc. Natl. Acad. Sci. U.S.A. **111**, 10.1073/pnas.1320670111 (2014).

PHYSICS

A Better-Known Electron Mass

The mass of the electron is one of the fundamental constants of nature. It is known to a high precision, but improvements are desirable in order to facilitate continued precision testing of the Standard Model of particle physics. The tiny mass, however, makes direct measurements very challenging. Sturm *et al.* use an indirect technique, in which an electron is bound to a carbon nucleus in a hydrogen-like configuration. This positively charged ion follows a circular orbit in an external magnetic field at the cyclotron frequency, which is proportional to the local magnetic field B .

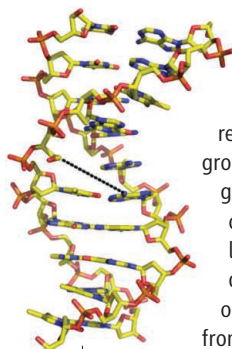
At the same time, the spin of the electron precesses at a frequency also proportional to B . By measuring the ratio of these two frequencies, one can determine the electron mass, knowing the mass of the ion and the g factor of the bound electron, which is different from its well-known free-space value; to estimate it, the authors used the theory of quantum electrodynamics and related measurements in a silicon system. The measurements were performed in a Penning-trap setup and yielded a relative precision of 3×10^{-11} in the electron mass, a value 13 times smaller than the uncertainty of the current accepted mass obtained by weighted averaging of literature values. It is expected that the improved result will enable future fundamental physics experiments that were previously impossible. — JS

Nature 10.1038/nature13026 (2014).

CHEMISTRY

Highly Reactive Abasic DNA

The base on a DNA residue can be removed to leave an abasic site. This reaction can occur spontaneously, can be catalyzed enzymatically during certain repair processes, or can be induced by certain anticancer drugs and mutagens. The sugar of the abasic site can have two different structures that are in equilibrium: a cyclic hemiacetal form and a ring-opened form that bears an aldehyde group. Price *et al.* report that if the abasic site is



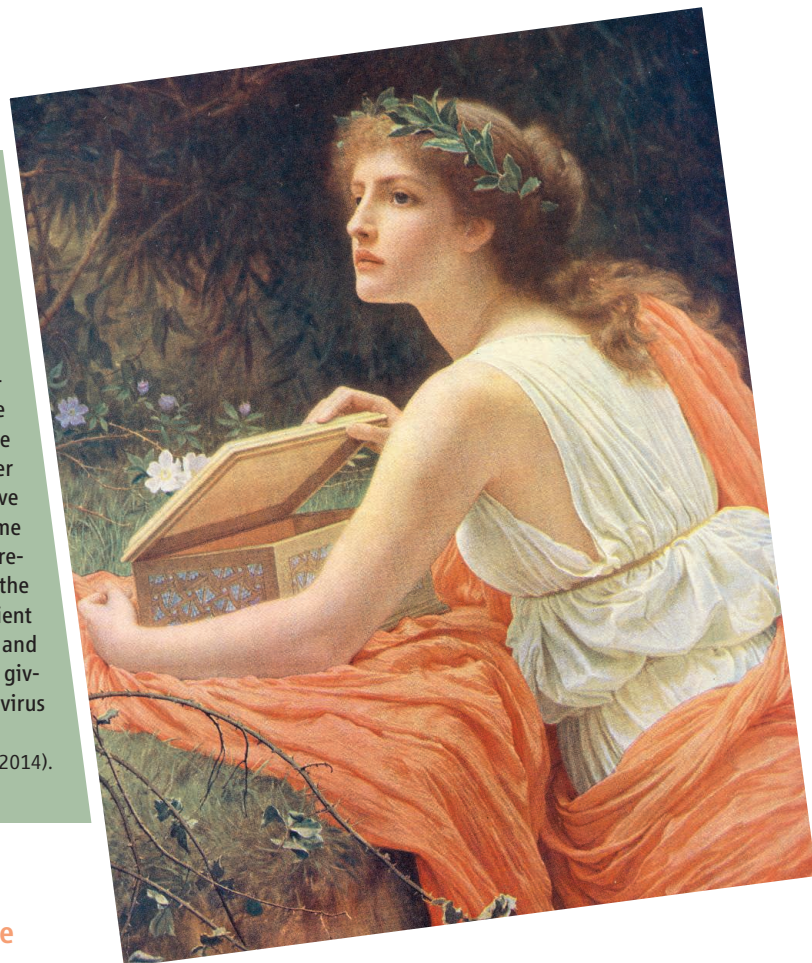
opposite an adenine residue on double-stranded DNA, the aldehyde group of the ring-opened structure can react with the exocycle amino group of the adenine (the N^6 -amino group) and form a stable covalent cross-link. The formation of cross-links, which are among the most deleterious of DNA mutations, occurs in remarkably high yields, from 15 to 70% under physiologically relevant conditions, versus the 2 to 3% yield observed previously for similar reactions with guanine residues. This difference arises because the N^6 -amino group of adenine is positioned in the major groove for adenine but the corresponding N^2 -group of guanine is in the minor groove. — PDS

J. Am. Chem. Soc. 10.1021/ja410969x (2014).

GENETICS

Aging Nucleosomes

DNA association with histone proteins to form nucleosomes provides barriers to transcription and replication and in this way provides careful regulation of these processes. As yeast age, histones are less prevalent and their overexpression is linked to increased life span. Given their regulatory nature, one might expect that a global loss of histones would result in increased expression of all genes; however, it has been reported that the expression of some genes goes up, whereas the expression of others goes down with aging. Hu *et al.* mapped nucleosome localization in aging yeast and revealed that nucleosomes became "fuzzier"—their positions became more variable along the DNA—and they showed a reduced periodicity, the tendency to recur at certain distances along the DNA. In contrast to studies showing increased repression of some genes during aging, a global loss of approximately 50% of nucleosomes correlated with the induction of expression of the entire genome. Furthermore, genes with the greatest induction contained sequences that more strongly assemble with nucleosomes. In addition to nucleosome loss and the induction of gene expression during aging, there was greater DNA damage at sites such as the ribosomal DNA locus and in mitochondrial DNA, as well as increased chromosomal trans



locations. Whether similar changes in nucleosomes are associated with aging in higher eukaryotes is yet to be determined. — BAP

Genes Dev. 28, 396 (2014).

CANCER

Metastasis in the Light

Cutaneous melanoma is less common than other forms of skin cancer but is far more deadly, especially if not detected at an early stage. Ultraviolet (UV) light from the Sun plays a key role in the initiation of melanoma by inducing mutations in the DNA of melanocytes, the pigment-producing cells in skin. A new study suggests that UV light does damage well beyond this early role: It appears to trigger a chain of pathologic events that facilitate metastasis.

Studying a mouse model, Bald *et al.* found that repetitive exposure of primary melanomas to UV light did not increase tumor incidence or growth but rather increased the number of lung metastases. Closer examination revealed that UV light induced the recruitment of certain immune cells (neutrophils) to the primary tumor. This inflammatory response in turn activated endothelial cells (cells that line blood vessels)



and triggered migration of the melanoma cells toward them, resulting in the expansion of tumor cells along blood vessels. This “angiotropic” growth pattern is thought to increase the likelihood that tumor cells enter the bloodstream and metastasize. Consistent with this, human melanoma samples with high levels of neutrophils were more metastatic. — PAK

Nature 10.1038/nature13111 (2014).

ATMOSPHERES

Synthetic Controls

Most of the anthropogenic warming of climate caused by greenhouse gas emissions is due to radiative forcing by carbon dioxide, the molecule that has garnered the lion's share of public attention. It is not the only emitted gaseous species that affect climate, however. Another class of compounds, long-lived synthetic greenhouse gases (SGHGs; gases with no significant natural sources and lifetimes of at least 1 year), has received far less attention although its constituents are responsible for a significant amount of warming, providing nearly 20% of the direct radiative forcing increase due to carbon dioxide since the preindustrial era. Rigby *et al.* examine recent trends in 25 of the most abundant SGHGs and construct emissions scenarios in order to estimate their future impacts on global warming. They find that if the relevant recommendations of the Montreal Protocol are implemented, overall SGHG radiative forcing would be reduced by approximately 26% by 2050, as compared to a policy without such controls, a cumulative reduction equivalent to 0.5 to 2.8 years of carbon dioxide emissions at current levels. — HJS

Geophys. Res. Lett. 10.1002/2013GL059099 (2014).

NEUROSCIENCE

Methylation Sees the Light

Animals, including humans, are sensitive to the length of cycles of light and darkness to which they are exposed. Disruptions of normal cycles by shift work or by exposure to bright lighting during normal periods of darkness can cause medical problems or disrupt learning and memory. Azzi *et al.* explored the mechanism by which mice responded to light/dark cycles that were shifted to be 22 hours long rather than 24 hours. This environmental change caused alterations in the expression of hundreds of genes in the superchiasmatic nuclei of the brain where the master circadian clock is located. Changes in DNA methylation in over 1000 regions of DNA that are associated with the change in light cycle were also observed. Affected genes included those encoding the clock components *Per2* and *Cry1*, where the changes in methylation were correlated with changes in transcription. Treatment of animals with an inhibitor of DNA methylation reduced the effects of the altered light cycle on the behavior of the animals. Thus, changes in DNA methylation at promoters or within the body of genes may mediate some of the effects of changes in light cycle on physiological function. — LBR

Nat. Neurosci. 10.1038/nn.3651 (2014).

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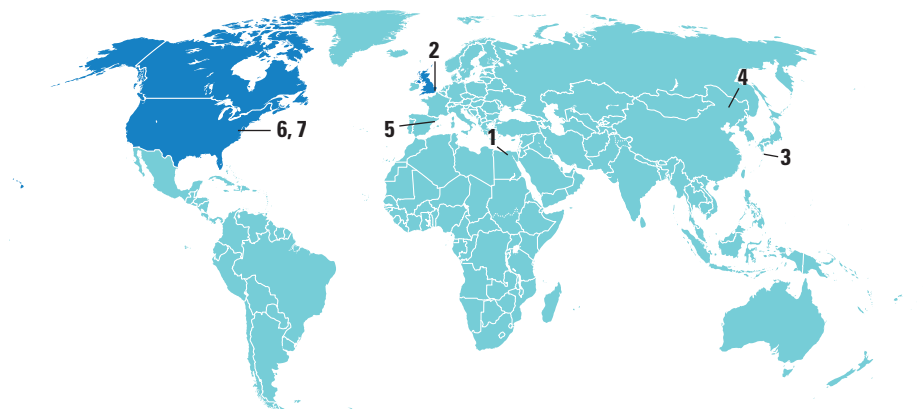
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AROUND THE WORLD



Cairo 1

MERS Found in African Camels

Middle East respiratory syndrome (MERS), the deadly viral disease discovered in Saudi Arabia in 2012, may be an African problem, too. Evidence suggests that camels in the Middle East are helping spread the disease, but researchers have now found it in camels shipped from Africa as well.

MERS has sickened 183 people and killed 80, most of them in Saudi Arabia.



Malik Peiris, an infectious disease researcher at the University of Hong Kong, and colleagues collected samples from four Egyptian abattoirs and found that most camels carried antibodies against MERS in their blood. They also found MERS RNA—a sign of current infection—in nose swabs from four animals shipped in from Sudan and Ethiopia. Peiris cautions that camels could have picked up the virus on their final journey in Egypt, but says that “health authorities really need to test patients with severe pneumonia all across Africa for MERS.” Another research team also reported last week that MERS is older than thought: Antibodies were present in camel serum samples from a Saudi archive going back to 1992. <http://scim.ag/AfricaMERS>

London 2

U.K. Proposes Regulations For ‘Three-Parent Embryos’

The U.K. government last week issued draft regulations that would let researchers try a controversial new in vitro fertilization procedure in patients. The technique could allow women who are carriers of mitochondrial disease to have healthy, genetically related children by transferring the DNA from their egg cells into a donor egg cell that has healthy mitochondria. Such altering of genes of human egg cells or embryos is currently forbidden in the United Kingdom.

The proposal would permit the procedure only for women who are highly likely to pass on the disease. The mitochondrial donor would have no claim to parental rights, and donors and recipients would be kept anonymous, unless both parties wanted to meet. After a public comment period through 21 May, the Department of Health will present a final proposal to Parliament. The procedure is also under scrutiny in the United States, where advisers to the Food and Drug Administration last week expressed concerns that it is not quite ready for human clinical trials. <http://scim.ag/mitoIVF>

Tanegashima, Japan 3

Researchers Eye Tethers For Space Debris

The Japan Aerospace Exploration Agency (JAXA) on 28 February launched a satellite to test a scheme to rid space of zombie satellites and other junk orbiting Earth. The 9-kilogram Space Tethered Autonomous Robotic Satellite-2 (STARS-2) is the first step toward a new debris-gathering technique. Scientists hope to use space robots to connect a long conductive wire to each



chunk of debris. Dragged through Earth’s magnetic field, the tether generates an electric current that dissipates as heat, draining kinetic energy from the system. This electrodynamic drag should pull debris into the atmosphere, where it will burn up. Over the next 3 to 6 months, STARS-2 will test tether deployment and electricity generation as a step toward future missions that will actually capture debris.

Developed by researchers at Kagawa University in Takamatsu, STARS-2 was one of seven university-designed microsattellites piggybacking on the launch of the Global Precipitation Measurement Core Observatory, jointly developed by JAXA and NASA to monitor rain and snow worldwide.

Harbin, China 4

Infamous Experimentation Site To Become Historical Park

Amid rising political tensions with Japan and other Asian neighbors, China announced this week that it plans to construct a “cultural park” on the site of Unit 731, where scientists under the Imperial Japanese Army carried out horrific experiments during the Second Sino-Japanese War and World War II.

The army established the covert unit, officially called the Epidemic Prevention and Water Supply Department, in Harbin in 1936. For nearly a decade, scientists there

NOTED

>Human genome sequencing pioneer J. Craig Venter has jumped with both feet into biomedical sequencing, this week [announcing his latest venture, Human Longevity Inc.](#) With \$70 million in startup funding, the company plans to study cancer patients, and later, centenarians and children, by sequencing their genomes and cataloging their microbiomes. The goal is to harness these data to make long-term predictions about health and ultimately improve preventive medicine.



Epic Slide

A global network of seismometers has located the largest known natural landslide since 2010. On 16 February, a sheer face of Mount La Perouse in Glacier Bay National Park in Alaska collapsed. That day, while checking a list of seismic events, geophysicist Colin Stark of Columbia University's Lamont-Doherty Earth Observatory in Palisades, New York, noticed a signal indicative of a landslide. Analyzing the signal using a technique Stark helped develop (*Science*, 22 March 2013, p. 1416), colleague Göran Ekström and postdoc Clément Hibert reconstructed how the face collapsed, slammed into the top of the glacier, then slid over the ice. Perhaps 68 million tons of rock, snow, and ice hurtled 7.4 kilometers, they estimate. This satellite image, 10 kilometers across, was taken on 25 February.

infected men, women, and children with plague, anthrax, and other biological agents and conducted vivisection without anesthesia. Thousands of Russians, Chinese, and other Asians died in the experiments.

After World War II, the U.S. government agreed to conceal what happened if the researchers shared the results of the experiments. The new park will feature artifacts from biological warfare tests, but bioethicist Jing-Bao Nie of the University of Otago, Dunedin, in New Zealand suspects it could become "part of a larger place for entertainment and tourism, rather than one in memory of victims."

Barcelona, Spain 5

Devastated Agency Foresees Slow Recovery

Spain's largest public research organization faces years of eroded research activity and a decreasing workforce, its leadership revealed in a grim report released 26 February. The Spanish National Research Council (CSIC) action plan for 2014 to 2017 outlined efforts to address a lack of positions and limited management flexibility.

After seeing its government funding slashed by 36% from its 2008 peak, the council neared bankruptcy in 2013. It has lost more than 2000 staff members since 2011, most of them on temporary contracts, and government restrictions have prevented CSIC from filling research posts as scientists retire. The agency intends to start repairing the damage by resuming its Ph.D. and postdoc recruitment

program, seeking government approval for a new tenure-track program, and helping scientists seek international grants.

Some Spanish scientists have commended the plan for being realistic, but critics worry that it fails to address the roots of the problem, including limited autonomy to pursue new research and a lack of strong leadership within CSIC institutes. http://scim.ag/_CSIC

Washington, D.C. 6

EPA Puts Hold on Copper Mine Permits

The U.S. Environmental Protection Agency (EPA) has halted the permit application for a large copper mine while it evaluates how to protect valuable habitat. The controversial Pebble Mine near Bristol Bay, Alaska, could produce as much as 300,000 metric tons of copper a year, but it threatens a fishery, worth \$1.5 billion a year, which provides one-half of the world's sockeye salmon.

An EPA report found that the mine would destroy or bury 68 square kilometers of habitat and endanger other areas. Under the

Clean Water Act, EPA lets the U.S. Army Corps of Engineers decide whether to issue a permit to harm wetlands. But the Pebble Mine is one of 30 cases where EPA has decided to evaluate the proposal itself.

Tom Collier, CEO of the Pebble Limited Partnership, called EPA's decision a "major overreach" based on inadequate research. The price of shares in Northern Dynasty Minerals, the sole remaining backer of the partnership, fell 30% after the decision was announced on 28 February.

Washington, D.C. 7

Obama Proposes Extra \$5 Billion For Science, but Strings Attached

President Barack Obama presented a \$3.9 trillion budget request to Congress on 4 March that includes about \$135 billion for U.S. government research programs in the 2015 fiscal year, which begins 1 October. Overall, the proposed budget would give just small increases to the National Institutes of Health, the National Science Foundation (NSF), the Department of Energy's Office of Science, and other heavyweight funding agencies. But it requests \$5 billion in additional spending for a number of initiatives, including 1000 additional research grants at NSF; a new biosafety research laboratory in Athens, Georgia; and a new high-risk, high-reward funding program for biomedical science modeled on the military's Defense Advanced Research Projects Agency. Congress, however, is likely to balk at the tax and spending changes needed to free up that money. See *Science's* ongoing budget coverage at <http://scim.ag/Budget15>.



Bristol Bay

CREDITS (TOP TO BOTTOM): ASTRIUM VIA APOLLO MAPPING; COURTESY OF COLIN STARK; BILL ROTH/ANCHORAGE DAILY NEWS/CMCT/LANDOV

NEWSMAKERS

Three Q's

Since segueing from a Ph.D. in theoretical physics to filmmaking, **Mark Levinson** has done post-production work on movies including *Cold Mountain* and *The Social Network*. But *Particle Fever*—his first documentary—combines the two loves. Released this week, the film follows six physicists through the breakthroughs and heartbreaks leading to the discovery of the Higgs boson in 2012.



Q: Why did you want to direct this movie?

M.L.: In fiction films, usually you see stereotypes of scientists, and you don't really see the excitement, the humanity, what really drives them. That was

something that I knew, and felt strongly about. ... The idea was to do a narrative, dramatic story.

Q: Was it hard to generate that kind of excitement from 4 years of particle physics?

M.L.: The ingredients were there. We didn't have to generate that. The actual develop-



ments on their own ended up creating a narrative that we never could have dreamed of.

Q: How did the scientists respond to having their work filmed?

M.L.: They were really very open. I think they trusted the fact that we were going to show them accurately. The one

requirement was, "Please, just don't make us look boring."

FINDINGS

Viruses on Ice for Millennia Still Infectious

Researchers have brought to life a giant virus dating back to the days of the last Neandertals. Previous projects have resurrected ancient pathogens, such as the 1918 flu virus, by reconstructing their genomes from old DNA, but "nobody has been able to use a virus that old to infect a host," says Marco Coolen, a molecular paleoecologist



THEY SAID IT

"I was drunk in the bubble I created."

—Korean stem cell researcher Woo Suk Hwang, whose 2009 conviction on embezzlement and bioethics violations was upheld last week, in a recent interview. <http://scim.ag/HwangCourt>

at the Woods Hole Oceanographic Institution in Massachusetts. Chantal Aberger and Jean-Michel Claverie from Aix-Marseille University in France simply dissolved 32,000-year-old frozen soil from Siberia and mixed in amoebas, known hosts for giant viruses. The newly discovered virus, called *Pithovirus sibericum*, killed the amoebas, which suggests that other pathogens can

last thousands of years in permafrost and may be let loose as the planet warms, the researchers report this week in the *Proceedings of the National Academy of Sciences*. *P. sibericum* is the biggest virus discovered to date, but has about half the DNA of other giant viruses. <http://scim.ag/icevirus>

Random Sample

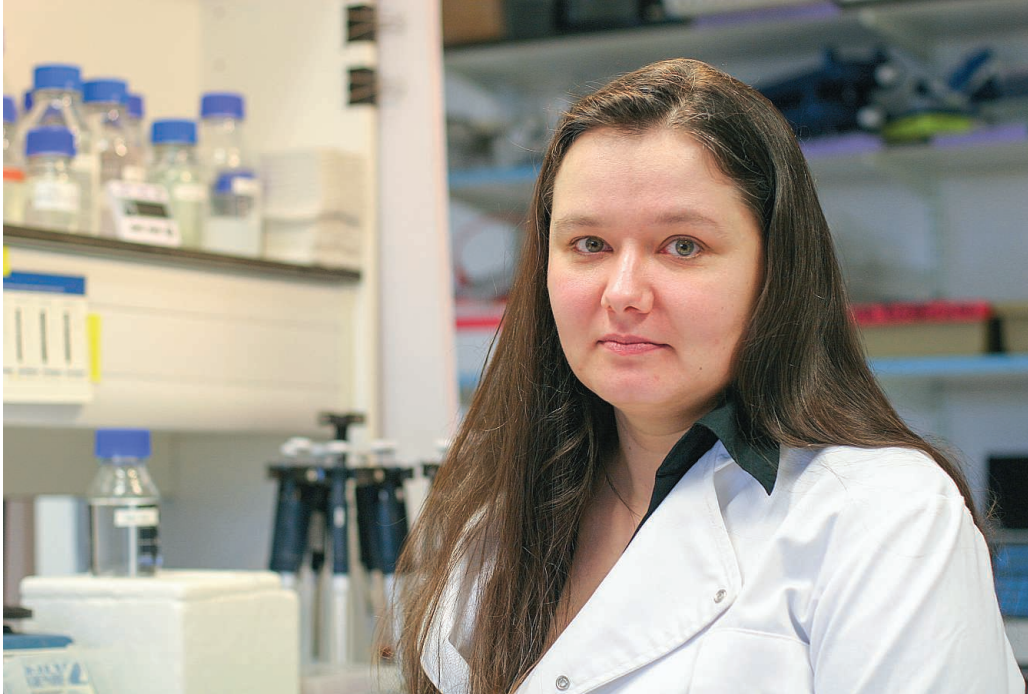


Largest Rodent Could Be Lab Rat for Stroke Studies

People undergo dramatic changes during puberty, but we've got nothing on the capybara. For reasons still mysterious to scientists, the world's largest rodent shuts off one of the main supplies of blood to its brain when it reaches sexual maturity. That makes the South American rodent an ideal natural model for studying stroke, a group of researchers in Brazil now proposes.

In both humans and capybaras, there are two main sources of blood to the brain: the internal carotid artery, which runs up each side of the neck, and the basilar artery, which begins at the base of the skull. When a capybara begins to sexually mature at about 6 months of age, its internal carotid artery becomes clogged with collagen and can no longer transmit blood. Still, "capybaras seem to cope very well" with the transformation, says University of São Paulo veterinary anatomist and surgeon Augusto Coppi. As the carotid artery closes, the rodent's basilar artery doubles in size to keep the brain supplied with blood.

When one of these arteries becomes blocked in a human, however, the other "doesn't have enough time to adapt," Coppi says—and the result is a stroke. In a recent paper in *Cells Tissues Organs*, he and colleagues argue that studying the capybara could help scientists figure out how to make human arteries behave more like their capybara counterparts and quickly pick up the slack if their companion shuts down.



From lab to courtroom. Magdalena Koziol claims her boss retaliated against her after a fellow postdoc tampered with her experiments.

statement that acknowledges the sabotage and says the culprit's employment was terminated immediately. But the university dismisses Koziol's complaints against her former boss and Yale and says that it "will mount a vigorous defense."

The complex case raises a host of questions about how to deal with sabotage, a type of misbehavior that some scientists believe is more common than the few known cases suggest. One key point of debate is whether ruining someone's experiments should fall under the definition of research misconduct, which is usually restricted to fabricating or falsifying data and plagiarism.

Some experts argue that wrecking experiments, while terrible, is more akin to slashing a fellow researcher's tires than to making up data.

Koziol declined to discuss the case with *Science* on the advice of her lawyer. Her complaint says that she first repeated her fish experiment to persuade Giraldez, who suspected the animals were poisoned with ethanol. Koziol told him she also had reason to believe someone had spiked her reagents. Giraldez and Robert Alpern, dean of the Yale School of Medicine, agreed to install the secret cameras that supposedly fingered Ocbina. (The complaint doesn't speculate about his possible motives.) Giraldez and Yale lawyer Howard Rose confronted Ocbina with the evidence on 8 March 2012, and he confessed, according to the complaint. At a lab meeting the next morning, Giraldez said Ocbina would not return to the lab and told his group not to discuss the incident. He also threatened Koziol with "legal consequences" and "prosecution" if she did, she claims.

From then on, Koziol's relationship with her boss deteriorated. The complaint says he refused to provide her with a letter about the sabotage, which presumably would have helped explain her lack of data to future employers. Koziol alleges that he criticized her work and character, didn't help her make up for the lost time, gave her "angry looks when passing in the lab,"

SCIENTIFIC MISCONDUCT

Sabotaged Scientist Sues Yale and Her Lab Chief

When Magdalena Koziol suspected that someone was sabotaging her research at Yale University, she did what comes naturally to a scientist: She set up a controlled experiment to test her hypothesis.

Koziol's studies of how the genome switches on after an egg is fertilized had begun failing mysteriously in July 2011, a month after she started her postdoc in the developmental biology lab of Antonio Giraldez. In August, she began producing transgenic zebrafish; they all died, not once, but time after time. A lab technician assured her she was doing everything right, and colleagues' fish were fine. So Koziol produced a new batch of fish and divided them in two groups. One she put in a container labeled with her initials, MK, as she had done before. She left the other half unmarked. Sure enough, the labeled fish died; the others were fine.

The experiment was a key step in proving that someone was tampering with her experiments, according to a lawsuit Koziol filed with the Superior Court in New Haven on 7 February. When hidden cameras were

installed in the lab, they revealed a fellow postdoc poisoning her fish, the complaint says. Now, Koziol is suing the alleged perpetrator, Polloneal Jymmiel Ocbina. According to the complaint, he left Yale after he was caught on video.

But Koziol, now at the Gurdon Institute at the University of Cambridge in the United Kingdom, is also suing Giraldez and Yale University. In her complaint, she alleges that after the saboteur was nabbed, Giraldez didn't allow her to speak about the affair, became increasingly hostile, and threatened to fire her. Koziol accuses him and Yale of negligent and intentional infliction of emotional distress and breach of contract. Among other things, she's asking for an unspecified amount of compensation for the lost time and funding—she had a grant from the prestigious Human Frontier Science Program Organization (HFSPO)—attorney fees, and emotional suffering.

Ocbina, who now works at a communications company in New York City, declined to comment because the case is in court; so did Giraldez. Yale sent *Science* a

didn't list her as a contributor to a *Nature* article, and threatened to fire and "destroy" her. Koziol became depressed, suffered from sleeplessness, and gained weight; when she and Giraldez talked for 3 hours in August 2012, Koziol "cried throughout the meeting," the complaint says.

Koziol filed a grievance procedure against Giraldez, which she lost; Yale, in its statement to *Science*, calls her allegations against Giraldez and the university "factually distorted and legally baseless." Giraldez's request to lab members not to discuss the case was "[i]n keeping with the law of the State of Connecticut, which protects the confidentiality of certain employment information," the university says. Lisa Rasmussen, a philosopher and research ethicist at the University of North Carolina, Charlotte, says it's not uncommon for misconduct cases to remain under wraps because the law requires a university to protect personal information about its employees. But Koziol's lawyer, Daniel Kryzanski, says that the university cannot restrict free speech about the reasons why someone was fired.

Publicly known incidents of sabotage in science are rare. The only recent one in the United States happened 4 years ago at the University of Michigan, Ann Arbor, where a postdoc named Vipul Bhrigu confessed to repeatedly killing the cultured cells of a colleague, Heather Ames, also using ethanol. He, too, was caught using hidden cameras. Bhrigu told a *Nature* reporter that he was under "internal pressure," and that he had hoped to slow Ames's work.

Theodora Ross, Ames's boss at the time, says that after the case became public, she heard from many people who suspected or knew of foul play in their own labs or elsewhere. "I think it happens a lot," says Ross, who's now at the University of Texas Southwestern Medical Center in Dallas. Sabotage isn't hard to commit, especially in biomedical labs, where samples and reagents are often stored in communal cabinets or fridges. And it's hard to detect or prove; plenty of experiments fail without anyone committing mischief.

Koziol's complaint also contends that Yale broke its contract with her by failing to report her case to the Office of Research Integrity (ORI), the U.S. agency that

investigates misconduct in federally funded biomedical research. In Bhrigu's case, that's what happened: The university reported the case to ORI, while the state of Michigan prosecuted Bhrigu, who pleaded guilty to malicious destruction of property and was sentenced to more than \$30,000 in fines and restitution.

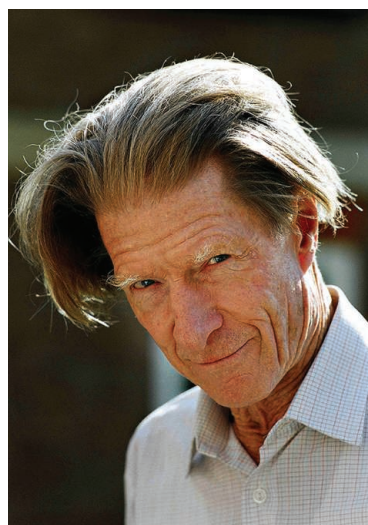
Kryzanski, Koziol's lawyer, says Yale didn't report the case to the police as a potential crime. Yale declined to specify how it has treated Ocbina's case, but its statement says that Giraldez notified the U.S. National Institutes of Health, which funded Ocbina's work and is one of the agencies under ORI's purview. An ORI spokesperson told *Science* that the office can "neither confirm nor deny" whether it was informed about the case.

Whether sabotage belongs under ORI's

"After Defendant Ocbina resigned or was terminated, Defendant Giraldez did not allow the Plaintiff and other members of his laboratory group to talk about the incident, and Giraldez even threatened the Plaintiff with 'legal consequences' and 'prosecution' if she were to talk about the incident. Defendant Giraldez also denied the Plaintiff documentation confirming that her fish had been poisoned."

Magdalena Koziol v. Yale University, Polloneal Jymmiel Ocbina and Antonio Giraldez

purview is questionable, Rasmussen says. A long and contentious debate took place in the 1990s over whether the U.S. federal definition of research misconduct should include anything beyond fabrication, falsification, and plagiarism, commonly referred to as FFP. Some argued that other types of bad behavior, such as sexual



Mentor. After leaving Yale, Koziol returned to the Cambridge, U.K., lab of Nobel laureate John Gurdon, who strongly supports her.

harassment or vandalism, could constitute research misconduct as well; others said that would open the floodgates to all kind of accusations, and that such misdeeds could be dealt with through other mechanisms.

In the end, ORI adopted the FFP-based definition. Yet it did issue a ruling in the Bhrigu case; in 2011, the agency ruled that his tampering "caused false results to be reported in the research record," and thus amounted to data falsification. The research record, in this case, was simply the lab notebooks in which Ames

recorded her failed experiments, Ross says; Bhrigu's obstruction didn't result in any flawed papers. It will be "interesting" to see whether ORI has gotten involved in the Ocbina case, Rasmussen says, because Koziol presumably mentioned the dead fish in her notebooks as well.

Koziol left Yale in March 2013 and returned to the lab of Nobel laureate John Gurdon in Cambridge, where she had done her doctoral work. "I was very happy to have her back," Gurdon says, "because her work is excellent. She was a model student." Gurdon helped secure a small grant for Koziol and donated some of his personal money to keep her going. He's optimistic about her chances against Yale. "They wrote her a letter promising her circumstances in which she could conduct her research," he says. "And they quite clearly did not provide even remotely adequate circumstances."

Gurdon has written HFSP, Koziol's funder, urging the program to withhold support for Yale if the university can't properly explain what happened. HFSP Secretary General Ernst-Ludwig

Winnacker says he sympathizes with Koziol, but does not know the details of her case. He says he had urged the parties to avoid an expensive and lengthy court fight. "It would have been much better if they had reached a compromise," Winnacker says. "It's too bad they couldn't."

—MARTIN ENSERINK

CREDIT: PHOTO BY JOHN OVERTON

VIROLOGY

A Bid to Thwart HIV With Shot of Long-Lasting Drug

A single injection of an anti-HIV drug may someday protect people from infection with the AIDS virus for up to 3 months. That's the implication of monkey experiments reported on page 1151 in this issue of *Science*. "This is the most exciting thing happening that I know of in HIV prevention studies today," says Robert Grant, a virologist at the University of California, San Francisco (UCSF), who was not involved in the studies.

A vaccine to prevent HIV infection is still the ultimate goal, says David Ho, whose team at the Aaron Diamond AIDS Research Center in New York City led the new monkey studies. But Ho says the long-lasting, injectable drug "may be able to fill that gap."

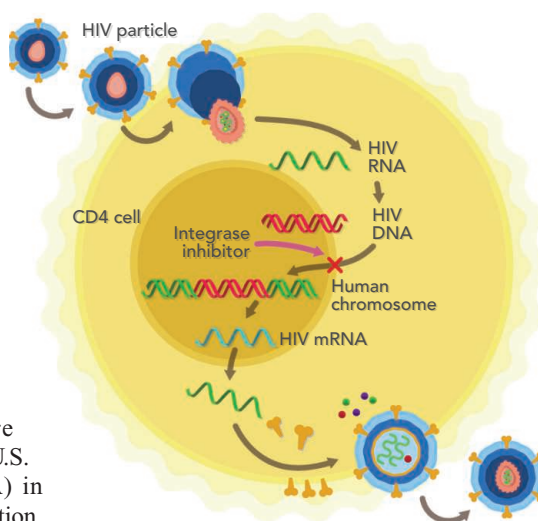
This novel approach builds on the proven strategy of protecting people uninfected with HIV using antiretroviral pills—so-called oral pre-exposure prophylaxis, or PrEP. Approved by the U.S. Food and Drug Administration (FDA) in 2012 for people at high risk of HIV infection, oral PrEP has one serious shortcoming: Pills prevent infection only if people take them every day. Grant, a pioneer of oral PrEP studies in humans, notes that healthy young people have been slow to adopt oral PrEP—and they account for 40% or so of the new HIV infections each year. "Receiving injections every 3 months would be a game-changer for them," he says.

Working with researchers from drugmaker GlaxoSmithKline (GSK) in Research Triangle Park, North Carolina, and the Tulane National Primate Research Center in Covington, Louisiana, Ho and his colleagues tested an experimental drug, dubbed GSK744, that inhibits an enzyme the virus depends on to integrate into human chromosomes and copy itself (see graphic). The integrase inhibitor is an analog of a GSK pill called dolutegravir, which the FDA approved in August 2013 to treat HIV infections. An offshoot of GSK, ViiV Healthcare, initially began developing GSK744 as an injectable treatment, not a preventative; small human safety and pharmacokinetic studies have been completed.

Ho says GSK744 has two "magical properties." First, it's insoluble, so at high concentrations

GSK744 forms crystals when suspended in a liquid. "When this nanosuspension is injected, it essentially creates a depot effect and the drug bleeds out at a predictable rate," Ho explains. GSK744 is also slowly metabolized (see graph).

An initial experiment first reported



Breaking the cycle. One GSK744 injection may disrupt infection for months by inhibiting HIV integration.

at the Conference on Retroviruses and Opportunistic Infections (CROI) in March 2013 showed that injections of GSK744 could give macaques long-lasting protection against a hybrid simian-human AIDS virus dubbed SHIV. The researchers gave two injections of the drug to eight macaques and then "challenged" the animals by putting SHIV into their rectums once a week. After eight challenges, none of the animals became infected. In contrast, SHIV readily infected eight untreated control monkeys. Next, the

researchers quantified how long a single dose could protect the animals. After injecting 12 macaques with GSK744 and waiting 1 week, they began weekly challenges with SHIV until the animals became infected. On average, the drug stopped working at the 10th challenge. Monkeys clear the drug much more quickly than humans do, so Ho and co-authors contend the protection should last even longer in people.

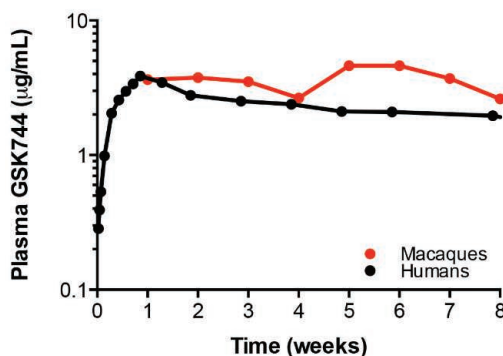
SHIV in monkeys, of course, may not reflect what happens with HIV in humans, and drug toxicities may surface. But a monkey model accurately predicted the effects of oral PrEP, and Ho says he is encouraged by the excellent safety profile of dolutegravir. (Ho's group and, separately, a research team from the U.S. Centers for Disease Control and Prevention, presented monkey data this week at CROI 2014 that show the drug works against vaginal infection, too.)

Philip Johnson of the Children's Hospital of Philadelphia in Pennsylvania thinks compliance could be a problem even with long-lasting PrEP. "This is going to require multiple injections over the lifetime of an individual," Johnson says. "How feasible is it truly in the long term?" Johnson is working on another PrEP approach that stitches a gene for a powerful anti-HIV antibody into a harmless virus that theoretically can produce it indefinitely. "Our goal is one injection, one encounter, and we're done," Johnson says. But he acknowledges that this gene therapy faces steeper regulatory hurdles than GSK744.

Salim Abdool Karim, an epidemiologist who heads the Centre for the AIDS Programme of Research in South Africa, is most excited by the prospect of combining GSK744 with other injectable antiretrovirals to simplify treatment, especially in hard-hit countries like South Africa. "The potential of these drugs is enormous for treatment," says Karim, who is based in Durban. As with PrEP, many people now receiving antiretrovirals have problems with adherence, potentially breeding drug-resistant strains of HIV.

UCSF's Grant says he's in discussion with ViiV Healthcare about helping run large-scale PrEP trials of GSK744 in people. He says studies likely will have to show only that the drug is safe and better than the oral PrEP now on the market, which means they could be completed within a few years.

—JON COHEN



Durable product. A GSK744 dose lasts for an unusually long time in both human and monkey blood.

NUTRITION

Diet Studies Challenge Thinking on Proteins Versus Carbs

A new theory about the foods that can extend life is taking shape, and it's sure to be a controversial one. Two studies out this week, one in mice and another primarily in people, suggest that eating relatively little protein and lots of carbohydrates—the opposite of what's urged by many human diet plans, including the popular Atkins Diet—extends life and fortifies health.

The research challenges other common wisdom, too. The authors of both studies believe that calorie restriction, a drastic diet that helps mice and other species live much longer than normal, may work not because it slashes calorie intake, but mostly because it cuts down on protein. They also speculate that the low-protein/high-carbohydrate balance that appears to extend life in the two studies, published in *Cell Metabolism*, could clarify why slightly plump people live longer on average than skinny ones—something epidemiologists have been hard-pressed to explain.

"If these two studies are really correct, what people in general are trying to do" to get and stay thin "might be completely wrong in terms of maintaining health and even longevity," says Shin-ichiro Imai, a molecular biologist at Washington University in St. Louis who studies aging.

The "if" is a big one, however. The interplay between diet and health is extraordinarily complicated, and protein diets in particular have sparked confusion. On the one hand, some researchers have found a correlation between consuming lots of protein and heart disease. On the other, high-protein diets have been linked to improved metabolic profiles, such as lower blood glucose levels. Teasing out the role of protein versus total calories or carbohydrates is no easy task. "There are a whole lot of variables here," says Cynthia Kenyon, who studies the biology of aging at the University of California, San Francisco.

An Australian group led by nutritional physiologist Stephen Simpson and biogerontologist David Le Couteur at the University of Sydney tried to clear up some of the confusion by assigning 858 mice to one

of 25 diets with different mixes of protein, carbs, fats, and fiber. All were allowed to eat as much as they wanted.

The mice whose diets included 5% to 15% protein and 40% to 60% carbohydrates lived the longest, up to 150 weeks compared with 100 weeks for those on a diet of about 50% protein. By comparison, Americans on average take in about 16% of their calories from protein. The animals on the low-protein/high-carb plan also had lower blood pressure, better glucose tolerance, and healthier cholesterol. (Levels of fat in their diet didn't seem to make much difference.)

Mice that ate lots of protein were skinnier—just as people on high-protein

10% or fewer of their calories from protein. The high-protein eaters were more than four times as likely to die from cancer over the 18 years after they were surveyed, and 75% more likely to die of any cause.

Those leaping to grab a breadstick instead of sausage should note, however, that as the NHANES cohort aged, protein became more important. In the over-65 crowd, those who ate lots of protein survived longer, on average, than those who ate less. Geriatricians, Longo says, have long extolled the value of protein in older people, and "they're right." Longo and Imai speculate that elderly individuals may be less likely to absorb the protein they take in, so need more of it.

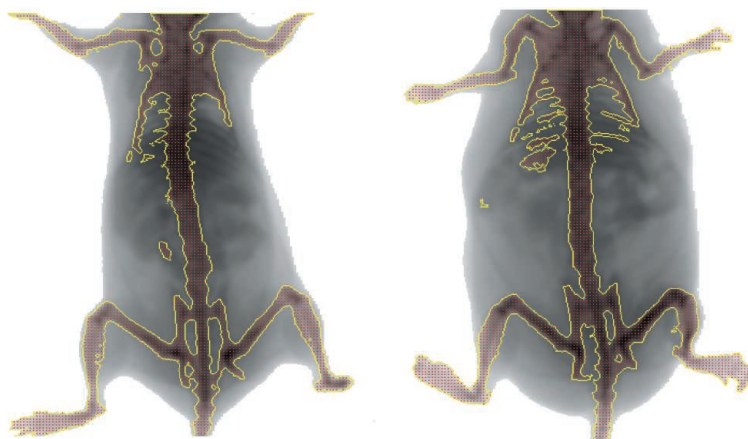
The findings do fit with some molecular clues. Cutting protein intake is known to reduce levels of a growth factor called IGF-1, and lower IGF-1 levels are linked to longer lifespan and reductions in the risk of cancer and diabetes. Limiting protein intake also reduces levels of a protein called mTOR, and lower mTOR extends life in mice. The Australians saw the mTOR effect in their animals. Longo's team, testing stored samples from the survey participants, saw that higher IGF-1

levels correlated with more dietary protein.

"There's certainly some truth to this relationship" between protein consumption and lifespan, says Matt Kaeberlein, a molecular biologist at the University of Washington, Seattle, who studies longevity. "But it's probably overly simplistic to say that everyone should go on a low-protein diet at this point." Among the many caveats, for example, is that the mouse study used a single strain, though different strains can have different reactions to diets such as calorie restriction.

Kaeberlein also thinks it's unlikely that reduced protein alone explains the dramatic impact of calorie restriction on lifespan. Le Couteur wants to find out for sure: He and his colleagues are planning a study that pits a low-protein/high-carb diet head-to-head with restricted calories, to see which mice live the longest.

—JENNIFER COUZIN-FRANKEL



Healthy weight. A skinny mouse (body x-ray scan, left) ate lots of protein and few carbs, but it wasn't as healthy or long-lived as its counterpart on a low-protein/high-carb diet.

diets tend to be. But for these mice, slender translated to ill health and earlier death. This finding, Le Couteur says, supports the "concept of healthy obesity," which has been raised by epidemiology studies of slightly overweight people. He suggests that if their diet is higher in carbohydrates and lower in protein than usual, that might be responsible.

The second study, led by gerontology researcher Valter Longo and graduate student Morgan Levine at the University of Southern California in Los Angeles, focused on data from 6381 adults over 50 years old who were interviewed once about their diet as part of NHANES, a national survey of health and nutrition. Longo's team used death records to conclude that those under 65 whose self-reported diets they classified as high-protein—the participants said at least 20% of their calories came from protein—were at much higher risk of illness and death than a group who took in



Global impact. Staff at Kayan Mentarang National Park in Borneo (left) and others use databases and mapping tools created by WWF scientists in Washington, D.C.

ENVIRONMENT

Major Conservation Group Guts Science Team in Strategy Shift

One of the world's largest and wealthiest conservation groups, the World Wildlife Fund (WWF), is dramatically shrinking its core research unit as part of a broad reorganization. The change will break up the WWF's storied science team, which had 30 staffers and a \$3.5 million budget, making it particularly big for a conservation nongovernmental organization. About a third of the Washington, D.C.-based researchers were laid off in late February and another third reassigned to other programs, WWF has confirmed.

The WWF move is "very bad news for science," says Thomas Brooks, who leads the science unit at the International Union for Conservation of Nature, in Gland, Switzerland. "I think the decision is short-sighted."

WWF's changes, however, echo earlier cuts in other large conservation science programs. The Nature Conservancy (TNC) spun off its informatics unit, NatureServe, in Arlington, Virginia, in 2000. Its remaining science staff shrank to a core unit of about 20 researchers. A decade ago, Conservation International had some 35 scientists working at its Center for Applied Biodiversity Science, but the team was downsized when \$35 million in funding from philanthropist Gordon Moore ran out in 2005. "The days of big science in NGOs are done," says Kent Redford of Archipelago Consulting in Portland, Maine, and former chief scientist of the Wildlife Conservation Society (WCS) in New York.

Tight money and changing priorities are driving the trend, say other observers.

The U.S. chapter of WWF stresses that the shift isn't a response to financial woes; its revenues are up by 28% since 2008, to \$229 million. Instead, WWF-US says it expects the restructuring to make it more agile at responding to conservation challenges. Jon Hoekstra, chief scientist and vice president at WWF-US, says the change will also encourage chapters in other countries to further build up their own science capacity. "I think it will dramatically expand the questions we can take on."

Founded in 1961, WWF works in about 100 countries, employs some 6000 people, and has a global budget of \$900 million. The U.S. chapter is the largest and has been an icon of conservation science. In addition to dozens of staff members with science degrees who work in field programs, a research unit in Washington, D.C., has operated since the mid-1980s. Over the years, it developed a classification of ecoregions, which is now widely used to set priorities for conservation. Researchers at WWF-US headquarters were also among the first to create detailed watershed models for planning and have advanced the science of freshwater biodiversity conservation.

In recent months, however, WWF-US decided to accelerate and scale up conservation efforts, especially in the southern hemisphere and Asia. The reorganization creates teams to work on six main goals: protecting species, forests, fresh water,

and fisheries; sustainably doubling food availability; and fighting climate change. Twelve of the central unit's scientists have been assigned to these teams. In addition, Hoekstra's position as chief scientist will be elevated to senior management. "Science is being put at the table for significant decisions at all levels of the organization," he says.

But another 10 scientists were shown the door by the end of February, victims of budget-cutting to pay for conservation. John Robinson, the current chief scientist at WCS, says that it's extremely difficult to fund a central team of scientists without major foundation help. "Eventually rational economics says, 'We can't afford this,'" he adds.

Hoekstra says he tried to preserve breadth of expertise across the staff. "We've made some changes that are really hard," he says. The eight remaining scientists will staff a smaller central unit for monitoring and evaluation of program goals, supporting a project to measure the economic value of ecological processes, a symposium series, and other tasks. Cost savings will go to a \$5-million-a-year innovation fund for trying out new ideas in conservation and research and to support other WWF chapters, including in Mexico and China.

Some of the slack will be taken up by WWF's new institute in Gland, which launched last year with a \$20 million grant from ornithologist and philanthropist Hans Lukas "Luc" Hoffmann, a co-founder of WWF. Director Josh Tewksbury says the idea is to bring in groups of outside scientists for short-term work on research issues relevant to the entire WWF network. This model provides more flexibility than having staff scientists, he suggests. In a similar project, TNC joined with WCS last year to create Science for Nature and People, based at an ecological think tank in Santa Barbara, California.

But some experts worry that by downsizing its central science unit, WWF-US may miss emerging issues and not have the critical mass to innovate new approaches and insights for conservation. "The role of science is to question, to speak truth to power," says Peter Kareiva, chief scientist of TNC. "When centralized, you have more authority to do that. You don't want to be too agenda-driven."

—ERIK STOKSTAD

CREDIT: TANTYO BANGUN/WWF-CANON

Chemical Atlas Shows Where Seas Are Tainted—And Where They Can Bloom

HONOLULU—About 1000 meters down in a remote expanse of the Atlantic Ocean sits an unusual legacy of humanity's love affair with the automobile. It's a huge mass of seawater infused with minute traces of lead, once widely emitted by cars burning gasoline laced with the toxic metal. Decades ago, the United States and Europe banned leaded gas and many other uses of lead, but the pollutant's fingerprint lingers on—as shown by a remarkably detailed atlas of digital maps unveiled here last week at the 2014 Ocean Sciences Meeting.

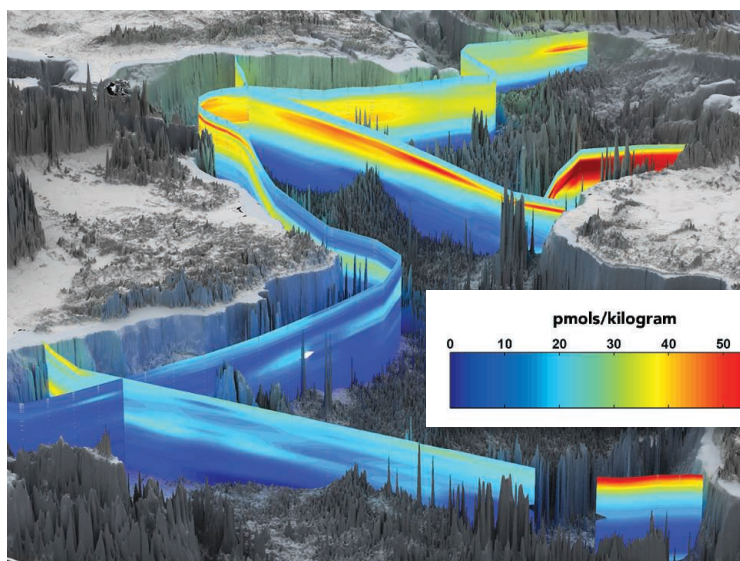
The atlas, which includes 3D maps and rotating animations, is the first product of GEOTRACES (geotraces.org), a \$300 million international collaboration to document the presence of key trace metals and other substances in the world's oceans. Researchers from dozens of nations gathered the initial release of data on about 30 cruises since 2010, collecting nearly 30,000 water samples at 787 study sites. Then, using painstaking techniques, which involved wearing "moon suits" and working in ultraclean laboratories to prevent contamination, they measured often minute quantities of more than 200 substances, both humanmade and natural.

The roster includes important "micronutrients" such as iron, which can fuel plankton blooms and influence how the ocean responds to climate change, and isotopes of carbon and thorium, which help scientists track the movements of seawater and the amount of dust that falls into the ocean. The breadth and accuracy of the data makes GEOTRACES "a huge improvement over what we were able to do in the past," says ocean chemist Hein de Baar of the Royal Netherlands Institute for Sea Research in Texel, a GEOTRACES participant.

Oceanographers began organizing GEOTRACES more than a decade ago. Existing studies were plagued by limited sampling and contamination of seawater samples from sources such as metal ship hulls and instrument cables. To overcome such

problems, GEOTRACES recruited dozens of teams to take samples from numerous depths and developed strict sampling and analytical methods. "Everything has to be precise and squeaky clean," says chemical oceanographer Christian Schlosser of the University of Southampton in the United Kingdom.

The resulting maps are letting researchers



Tainted. Red and yellow areas highlight areas in the Atlantic Ocean that have relatively high traces of lead from decades of pollution.

"see things that we couldn't see before," says chemical oceanographer Abigail Noble of the Massachusetts Institute of Technology (MIT) in Cambridge.

The lead images, for example, tell a sobering story of past pollution—and continuing contamination. That mass of lead-tainted seawater deep in the central Atlantic started at the surface decades ago, where it collected airborne lead particles, Noble says. As the surface water slowly sank into the deep ocean, it became a time capsule recording "the incredible impact that we have had on the oceans in the past, and how it changes over time."

Although the elevated lead levels stand out as red blotches on the GEOTRACES maps, the concentrations pose little threat to humans or wildlife—at roughly 12 parts per trillion, they are far lower than the 15 parts per billion that the U.S. government considers to be of concern in drinking water. "You probably aren't going to see stupid fish or whales swimming around," says MIT ocean

scientist Edward Boyle, alluding to the brain damage that can be caused by lead exposure. And lead levels in much of the Atlantic have dropped dramatically over the past few decades, mostly thanks to the lead phaseout in the United States and Europe.

Still, the maps show that lead contamination continues in some parts of the world. Off the southern tip of Africa, surface waters with relatively high traces of lead are flowing into the Atlantic from the Indian Ocean. That's probably due to the continuing use of leaded gasoline in parts of Africa and Asia. Another hot spot

is where the Mediterranean Sea empties into the eastern Atlantic. The lead concentrations there "are some of the highest we saw anywhere" in the Atlantic, says chemical oceanographer Rob Middag of the University of Otago, Dunedin, in New Zealand. That may be because the Mediterranean is a relatively enclosed body of water with heavily settled shores and has collected pollution for centuries.

Other maps highlight the distribution of micronutrients such as zinc and cadmium, which can fertilize plant growth and will help determine how much planet-warming carbon dioxide the sea can soak up. And a few clearly reveal features such as the plumes of trace metals pumped into the deep sea by hydrothermal vents, or how dust blowing off Africa's deserts amplify surface levels of iron in nearby seas.

This detailed picture of ocean chemistry also raises new questions. Charts of isotopic data, for instance, suggest that researchers are underestimating the amount of dust that falls into certain parts of the ocean, potentially undermining computer models that use isotope data to help reconstruct ocean circulation patterns in the past. GEOTRACES is forcing researchers to find "lots of ways to politely say the old data was wrong," De Baar says. "Many old papers are now going to be primarily of historic interest." And this is just the beginning. As new cruises are completed in the coming years, particularly across the Pacific and Southern oceans, the GEOTRACES data trove will continue to grow.

—DAVID MALAKOFF

SPACE

Europe's Copernicus Offers a Daily Dose of Earth Data

On 8 April 2012, thousands of environmental and climate researchers around the world found their supply of data suddenly and unexpectedly cut off. Envisat, Europe's bus-sized Earth observation satellite, had fallen silent, and despite furious efforts by the European Space Agency (ESA) to reestablish contact, the spacecraft remained quiet. Data sets 20 years long, provided by Envisat and its predecessors, were cut short, interrupting a wide range of activities, from monitoring pollution at sea to gathering evidence for climate change. "It was a big loss," says Chris Steenmans, head of monitoring, data, and innovation at the European Environment Agency (EEA). "We had to rely on input from other spacecraft, but time series were broken."

Early next month, the first satellite in a fleet designed to continue the legacy of Envisat will lift off from Europe's spaceport in Kourou, French Guiana. The new program, called Copernicus (formerly GMES, Global Monitoring for Environment and Security), will avoid betting everything on a single spacecraft. Instead, Copernicus will spread its instruments over a half-dozen craft that will be launched over the next 7 years. Each of these so-called Sentinels will be followed within a couple of years by an identical copy. These B-units will increase the rate of data gathering and serve as backups. Later, C-units will be built to replace any failed satellites.

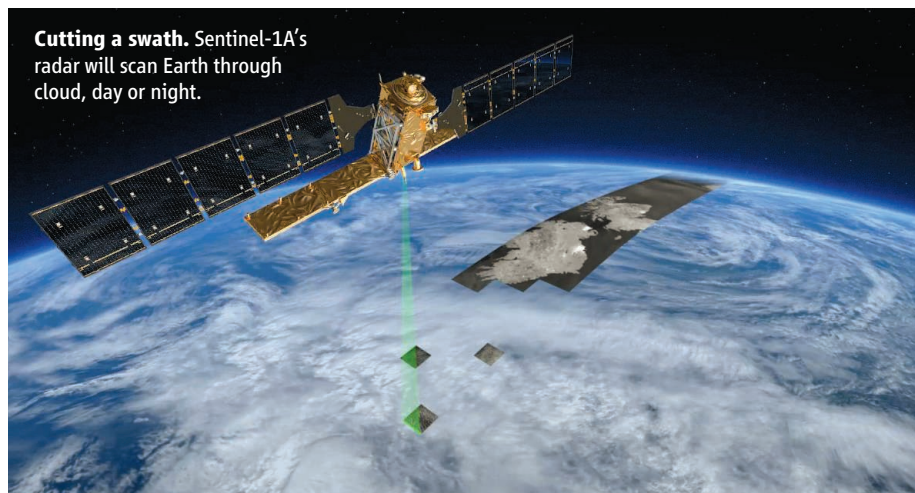
The result will be the world's first operational environmental monitoring system, a cousin to the operational meteorological services that have provided weather forecasts for decades. The sensors on the Sentinels will measure the state of the ocean surface and its temperature, monitor changes in land use, and take soundings of the atmosphere's composition and temperature.

Similar measurements have been made before by science satellites, but Copernicus aims to turn the process into an operational service: Each spot on Earth will be surveyed every few days, data will be supplied quickly in easy-to-use forms, and it won't be interrupted by the loss of a satellite. "It could be a game-changer," says EEA Executive Director Hans Bruyninckx. John Remedios of the University of Leicester, who's slated to head the United Kingdom's National Centre for Earth Observation starting in October, agrees. "We will get a very sophisticated understanding of our own continent," he says.

Sentinel-1A, due for launch on 3 April,

is a radar satellite that can image Earth day and night and can see through cloud. When Sentinel-1B joins it in 2016, the pair will map the whole Earth every 6 days. Their data will be used to detect and track oil spills, map sea ice, monitor changes in land use, and help emergency services respond to disasters. Sentinel-2A will join 1A in 2015 with sensors tuned to monitor forests, crops, and soil,

have to adjust our planning." The first launch was delayed from summer 2013. Copernicus also fared poorly in last year's negotiations over the next 7 years of the E.U. budget, coming away with €2 billion less than the €5.8 billion the European Commission had asked for. That will force ESA and the European Union to push back plans for an upgraded second generation of spacecraft,



Cutting a swath. Sentinel-1A's radar will scan Earth through cloud, day or night.

Mission	Launch	Purpose
Sentinel-1A	3 April 2014	Radar imaging for land and ocean services
Sentinel-2A	2015	Multispectral imaging for land monitoring
Sentinel-3A	2015	Multi-instrument mission for ocean forecasting and environment and climate monitoring
Sentinel-4A	2021 on EUMETSAT satellite	Atmospheric monitoring instrument
Sentinel-5P	2015	Atmospheric monitoring mission; bridges gap until launch of Sentinel-5
Sentinel-5A	2021 on EUMETSAT satellite	Atmospheric monitoring instrument
Sentinel-6A (Jason-CS)	c. 2020	Altimetry mission

followed by other Sentinels watching oceans and atmosphere (see table). Some Sentinels are not missions in their own right but instruments that will be added to spacecraft operated by EUMETSAT, the European weather satellite service.

A partnership between ESA and the European Union, Copernicus has spent €2.7 billion developing and building the A- and B-units of all six Sentinels. But it has suffered from delays both in satellite construction and in the ground infrastructure that will convert raw data into products for users in government, industry, and research. As a result, Steenmans says, "we constantly

to follow the C-units sometime next decade.

In the meantime, Copernicus is expected to make a big difference to a wide range of users—including climate scientists, maritime safety agencies, border guards, and urban planners—because it emphasizes providing data that are usable, regular, and reliable. "It feeds into our core work, but we're also making an effort to make data available in user-friendly ways for other communities," Bruyninckx says. Remedios sounds a similar note: "It's a step forward in the way we use science and the way we help society to use science."

—DANIEL CLERY

Structural Biology Scales Down

The United States is winding down a \$1 billion project to churn out protein structures. What will that mean for the field?

THREE YEARS AGO, ANDRZEJ JOACHIMIAK decided to take on the superbugs. Infections from these antibiotic-resistant microbes are on an alarming rise globally, accounting for 2 million cases and 23,000 deaths a year in the United States alone. Among the most dangerous bugs are new strains with a protein known as NDM-1 that chops up a wide variety of previously effective antibiotics known as β -lactams, drugs that include penicillin.

Thanks to a long-running effort called the Protein Structure Initiative (PSI), Joachimiak had the tools to work out NDM-1's structure and pinpoint its weaknesses. Joachimiak, a structural biologist at Argonne National Laboratory in Illinois, and his colleagues used robots to synthesize 98 NDM-1 genes, each with subtle sequence variations. They succeeded in engineering bacteria to express 59 of those genes and produce their corresponding proteins at a high concentration. The researchers purified 53 of the proteins and coaxed 21 into forming crystals, many in combination with different druglike inhibitors and potential antibiotics. Then they shipped the best samples to the Advanced Photon Source, a stadium-sized synchrotron that fires a powerful beam of x-rays, bouncing them off crystal-line solids to map their 3D atomic structures.

Joachimiak and his colleagues worked out 11 such atomic maps; others are still in progress.

So far, the maps have shown that NDM-1 has an enlarged, flexible active site that allows it to fit, and ultimately break down, a wide variety of β -lactam antibiotics. Now, drug companies around the globe are free to use the results to design novel antibiotics that, someday, may save millions of lives. It was the PSI at its best, Joachimiak says.

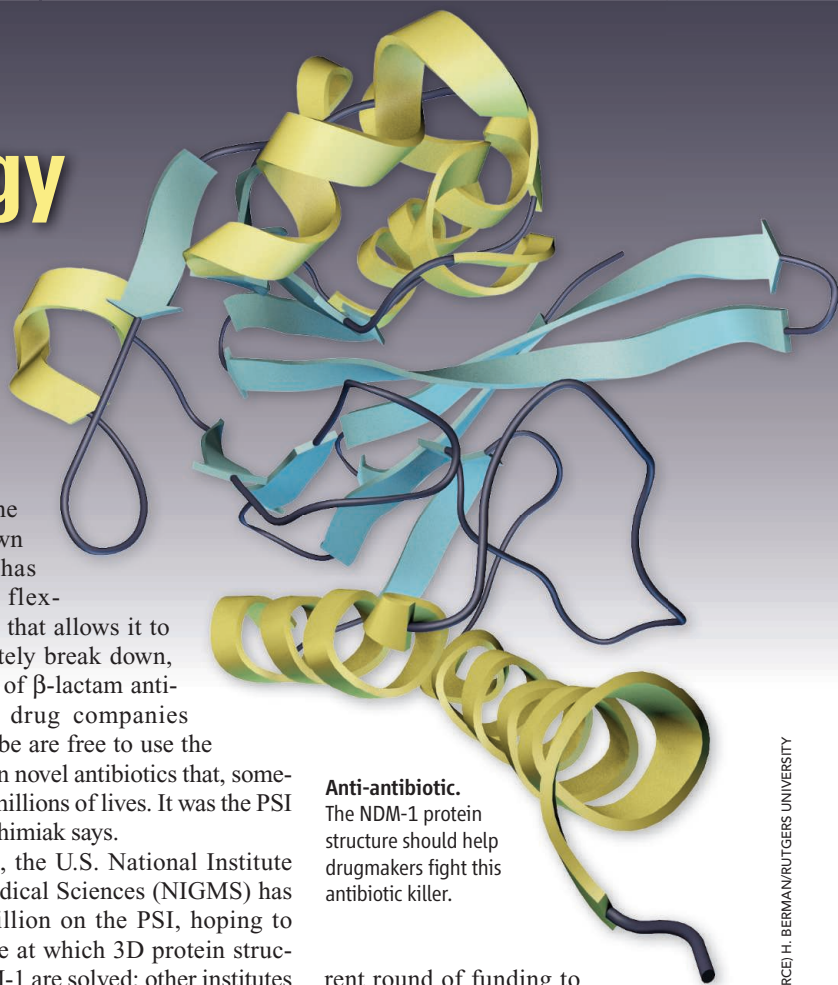
Since 2000, the U.S. National Institute of General Medical Sciences (NIGMS) has spent \$907 million on the PSI, hoping to rev up the pace at which 3D protein structures like NDM-1 are solved; other institutes of the National Institutes of Health (NIH) chipped in another \$23 million. That money funded large teams of biologists, physicists, chemists, and engineers to collaborate on not only determining protein structures, but also reinventing the way that this science is done. So far, PSI investigators have worked out the structures for 6507 proteins, 6.6% of all the structures with 3D data deposited in the international repository known as the Protein Data Bank (PDB).

But last fall, an NIGMS advisory council bowed to long-standing criticism of the PSI and pulled the plug on it, allowing its current

Anti-antibiotic. The NDM-1 protein structure should help drugmakers fight this antibiotic killer.

round of funding to expire in June 2015. "In the current budget environment, in order to start a new program or bolster support for existing priorities such as investigator-initiated research, other programs must be adjusted or ended," NIGMS's new director, Jon Lorsch, wrote in a blog post in September 2013.

The announcement left longtime supporters of the PSI reeling and critics gleeful. But most of all, it has raised a string of questions: What was learned from the near \$1 billion big-science experiment? What will happen to this team-oriented approach to biology? What will become of the high-speed facili-

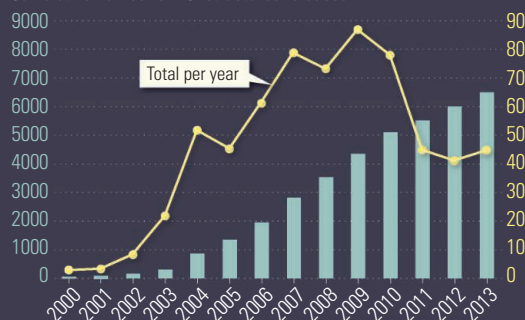


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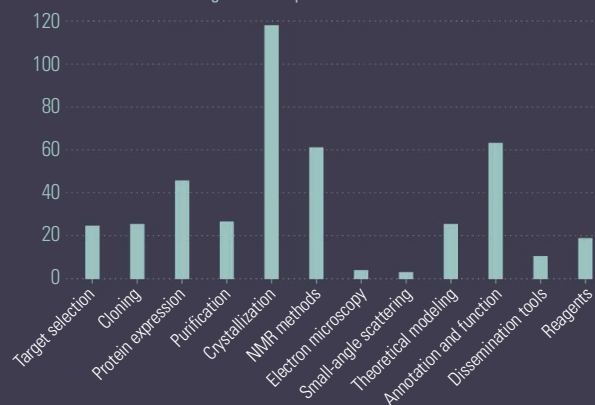
PSI's History In Numbers

Ups and downs. The PSI cranked out protein structures and technologies. But rising costs squeezed competitive research grants.

Cumulative number of PSI structures released



Number of PSI technologies developed



ties that were created? And what does the PSI's demise mean for the future of structural biology in the United States? "Structural biology really is at a crossroads," says Raymond Stevens, a structural biologist at the Scripps Research Institute in San Diego, California, and the leader of a PSI center devoted to solving structures of cell membrane proteins. "The PSI is dead. I view it as an opportunity to think about what's next."

The hunt is on

Before the PSI, structural biology was painfully slow. Typically, individual labs worked for months or years to clone a gene for a particular protein into bacteria or yeast cells and purify it. Then they often tried adding countless combinations of salts, buffers, and other additives to their protein-laced solutions to coax the proteins to arrange themselves into tiny crystals. The good ones could then be blasted with x-rays to see whether they would diffract in a tight pattern. After that, researchers often spent additional months or years mapping out the atoms. By the late 1990s, the PDB contained structures of only about 10,000 proteins. Meanwhile, the Human Genome Project was about to inundate researchers with genes for all the million-plus proteins in the human body. Determining their 3D structures would be a key step in sorting out their functions—and biologists realized that they would have to pick up the pace or fall hopelessly far behind.

Enter the PSI. In 2000, NIGMS officials laid out the program's goals. First, develop the technology needed to solve 5000 structures in 10 years. Then learn how to bypass crystallography altogether by using the solved structures to develop computer models that could take the gene sequence of an unknown protein and compute its likely 3D shape, giving insights into its function.

From September 2000 through June 2005, NIGMS spent \$265 million on a pilot

program, automating each phase of protein structure determination, including expressing proteins, purifying them, crystallizing them, collecting diffraction data at synchrotrons, and using software to solve their structures. More than 1100 structures later, NIGMS officials decided that the effort had succeeded well enough to push for a second "production" phase, PSI-2. From July 2005 through June 2010, NIGMS spent \$346 million on four large-scale high-throughput centers, six specialized centers focused on developing methods for solving more challenging structures, and a pair of computer modeling centers. All told, the effort generated another 3700 structures. Most were unique, meaning that they shared less than 30% of their genetic sequence with any other protein and folded in ways no other protein did.

But the PSI also churned out controversy. The bulk of the newly discovered proteins came from bacteria, and researchers knew little about their function. PSI researchers argued that the bacterial proteins were teaching them basic rules of protein folding. But biologists outside the PSI wondered why so much effort was being spent pursuing proteins unlikely to improve human health. In 2007, a midterm review of the PSI-2's progress, led by University of Michigan, Ann Arbor, structural biologist Janet Smith, concluded that "the large PSI structure-determination centers are not cost-effective in terms of benefit to biomedical research." The reviewers recommended that the PSI be revamped to target proteins of high interest to biologists.

NIGMS obliged and funded a third phase of the program, dubbed PSI:Biology. Gone was the talk of seeking out unique ways in which proteins fold and obtaining structures

of representatives of each protein "family." Instead, the four high-throughput centers and an additional nine centers refocused their efforts on solving biologically important structures.

Still, criticisms persisted. In a midterm evaluation of the PSI's third phase produced last year, yet another outside panel of biologists faulted the high-throughput centers.

"[M]any of the projects being developed are technology driven, chosen because they can capitalize on the existing high-throughput structure pipelines, rather than being driven by biological interest or impact," the report stated. The panel recommended continuing PSI:Biology for another 3- or 5-year term beyond 2015. But it also advised NIGMS to begin thinking about how best to end the program and move structural biology away from a dedicated source of set-aside funding.

Crystallography at 100

See also the special section on crystallography starting on page 1091.

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of representatives of each protein "family." Instead, the four high-throughput centers and an additional nine centers refocused their efforts on solving biologically important structures.

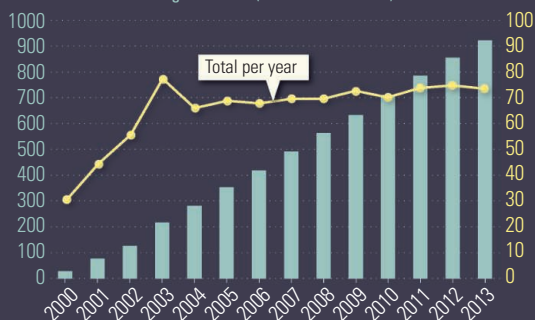
Disputed legacy

Lorsch and an NIGMS advisory panel jumped at the recommendation. They decided to forgo another phase and prepare right away for the transition, creating panels to work out what to do with the current PSI centers and all the equipment and technologies they have produced, and how best to fund structural biology going forward.

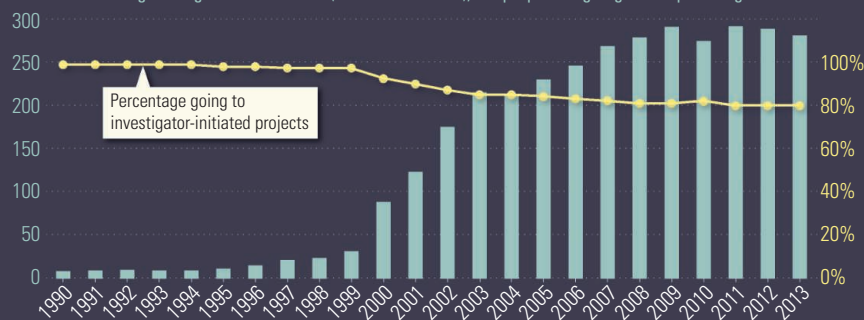
Opinions about NIGMS's decision are mixed. "The PSI was a bad idea from the start," says Stephen Harrison, a structural biologist at Harvard University and a longtime critic of the PSI. The initiative did speed technology development, he says, but much of that progress probably would have taken place anyway. Now that the program is being terminated, Harrison says, "structural biology can now go on where it should have gone all along": awarding grants to

CREDITS: (DATA SOURCE) NIGMS

Cumulative PSI funding from NIH (millions of dollars)



As NIGMS funding for large centers soared (millions of dollars), the proportion going to competitive grants declined.



projects deemed most valuable by conventional peer review.

Joachimiai says such criticisms are too facile. According to one estimate, the cost of producing the structure for one of the easier “soluble” bacterial proteins has plunged about 56% since 2003 to about \$50,000 per structure. A good chunk of the high-speed robotics and software that PSI labs developed for protein expression, purification, crystal growth, and x-ray structure determination are now in standard use by structural biology labs around the world. According to Helen Berman, an x-ray crystallographer at Rutgers University in Piscataway, New Jersey, who runs both the PDB and a PSI archive known as the Structural Biology Knowledgebase (SBKB), the PSI has produced 421 different technologies that have been either commercialized or disseminated through the SBKB online.



Built for speed. The PSI revolutionized a host of technologies, including robotic systems like this one for generating protein crystals en masse.

Beyond technology, Joachimiai and others argue that the PSI has made fundamental contributions to protein science. For example, Ian Wilson, a structural biologist at Scripps, and his colleagues have used the suite of tools at their high-throughput center to determine the structures of a large number of HIV and influenza viral proteins. Their goal is to identify common features in the proteins from each virus, which could provide targets for novel vaccines that would stop a wide variety of viral strains at once, rather than the one or two strains hit by current vaccines. And David Baker, a computational biologist at the University of Washington, Seattle, has used dozens of structures of stripped-down “ideal”

proteins solved by the Northeast Structural Genomics Consortium—a PSI effort—to sort out rules for designing novel proteins never made by natural organisms. Baker and colleagues are now using those rules to design synthetic proteins to serve as gene therapy agents, catalysts for converting carbon dioxide into fuel, and a host of other applications.

But Michigan’s Smith says projects such as Wilson’s HIV work and Baker’s protein design would have thrived anyway in a competitive funding environment of individual investigator awards, known as R01 grants. Meanwhile, she says, “there are a lot of problem-based structural biology projects of very high merit that are not getting funded right now, because there is not enough money.” If NIGMS redirects some of the money now spent on the PSI into investigator-initiated grants, “this will be positive,” she says.

Critics also fault the PSI for failing to identify enough rules of protein folding so that structures can be computed from their sequence, rather than laboriously solved. “There is no doubt that if you have a close [gene] sequence homology then you can do a lot of successful modeling,” says Michael Levitt, a computational biologist at Stanford University in California. However, he adds, “protein folding has not yet been solved generally.” PSI investigators concede the point. “Our computational methods still aren’t strong enough yet,” Stevens says. Levitt adds that even though PSI investigators have produced thousands of protein structures, the number of gene sequences encoding unknown proteins has grown much faster,

to more than 30 million. As a result, Levitt says, “it would take a very long time and an enormous amount of money” to solve structures of representatives of a large percentage of protein families.

Such brute-force efforts are now off the table, and the current PSI centers will be dismantled over the coming years. “The question is, how can we make this transition as orderly as possible with minimal collateral damage?” says Smith, who serves on the panel of outside experts advising the PSI on how its assets should be distributed. One option is for NIGMS to continue to fund high-throughput protein expression, production, and crystallization facilities as centralized resources for the whole structural biology community to use. Another is to distribute some of these facilities and technologies among current structural biology labs. These high-speed tools “shouldn’t just go away,” Smith says. NIGMS hopes to decide between May and December of this year, after the panels are expected to submit their recommendations.

PSI investigators say dismantling their centers could imperil U.S. leadership in structural biology. “A lot of jobs will be ending,” Stevens says. “We’ll see a very significant drop-off in the number of protein structures coming from the U.S.” Meanwhile, other countries, notably China, are ramping up their own efforts in high-speed structural biology. “I’m worried,” Joachimiai says. “We’ve made incredible progress. Now we’re looking at just shutting it down.” Wilson agrees. “We need a balance” between R01-type work and larger scale projects, he says.

But Douglas Sheeley, a program officer at NIGMS who is overseeing the work of the two PSI transition panels, says the PSI’s termination does not mean the agency is ending its support for structural biology or the collaborative team-based science that the PSI promoted. In 2012, NIGMS spent \$164 million to support structural biology, roughly 70% of the NIH total.

That total will almost certainly go down, because it includes \$75 million for the PSI. But Harrison insists that the U.S. structural biology community will thrive without the dedicated funds. NIGMS officials are considering using the PSI’s budget to fund an increasing percentage of R01-type grants. Even if that money no longer supports structural biology, “I think that’s okay,” Harrison says. It will force all structural biology projects to justify their merit against all other research. “We should compete on an even playing field.”

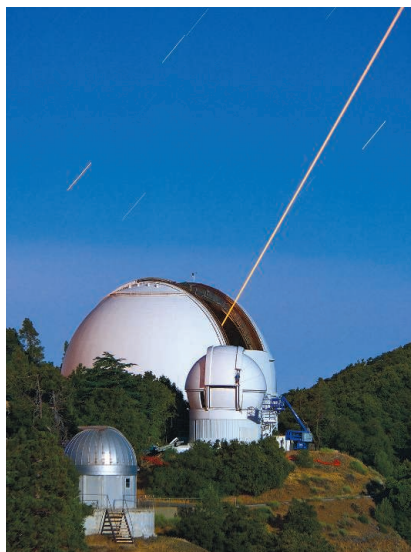
—ROBERT F. SERVICE



LETTERS

edited by Jennifer Sills

Small Telescopes, Big Rewards



Lick Observatory

THE CLOSING OF LICK OBSERVATORY (“LICK Observatory in trouble as austerity starts to bite,” Y. Bhattacharjee, *News & Analysis*, 13 December 2013, p. 1299) and all the major national optical telescopes on Kitt Peak National Observatory (“Money woes cloud future of workhorse U.S. telescopes,” Y. Bhattacharjee, *News & Analysis*, 20 December 2013, p. 1425) should hardly come as a surprise to astronomers. For many years, we have argued to funding agencies, often through prestigious decadal surveys [e.g., (1)], that to do the most important astronomy, only the largest and most expensive telescopes would suffice. Now that the funding agencies believe us, we are facing the consequences. The endgame of advocating increasingly expensive instruments has already been demonstrated by particle physicists with the cancellation of the Superconducting Super Collider in 1993.

Many of the greatest discoveries in astronomy (2) in the 20th century were made with small telescopes of their time. Optical pulsars (3), radio pulsars (4), and exoplanets (5, 6) were all first observed with small telescopes. Some discoveries made with large telescopes could have been made with small telescopes [such as quasistellar objects (7)], and others were made by both large and small telescopes [such as Sco X-1 (8, 9)]. The initial evidence for dark matter came from an 18-inch telescope (10). With the advent of affordable, back-illuminated charge-coupled devices, a 1-m telescope can explore an order of magnitude more of the universe than could the Palomar 200-inch telescope (now considered a candidate for small-telescope funding) in the photographic era.

Astronomers are getting what they asked for: A very limited number of telescopes so large and expensive that the workhorses of astronomy, the smaller telescopes that can afford to do the riskier and potentially more rewarding science, have been sacrificed. Lick Observatory, one of the most prestigious in the world, and all the important optical telescopes at Kitt Peak National Observatory are not the first. The smaller telescopes at Kitt Peak National Observatory and Lick had already been shuttered. More ominous, along with the hardware, the astronomers and students at the many smaller institutions are being sacrificed as well.

WILLIAM BRUCE WEAVER

Monterey Institute for Research in Astronomy, Marina, CA 93955, USA. E-mail: bw@mira.org

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A Safety Net for Diabetics

D. CLERY'S NEWS FOCUS STORY “A PANCREAS in a box” (10 January, p. 133), which discusses progress toward an artificial pancreas, outlines the fervent wishes of all but articulates a view of the future that potentially overlooks the “good” in a quest for the “perfect.” Industry is still perfecting sensors that have adequate sensitivity to safely drive an insulin pump unattended by human oversight. Contrary to Clery's assertion that continuous glucose monitor (CGM) sensors replace fingerstick readings, today's CGM sensors are considered to be “adjunctive devices.” FDA labeling of such devices typically reads: “[product ...] does not replace the information obtained from a standard home blood glucose meter but rather, it is used to complement the information obtained from the blood glucose meter” (1). A particular challenge for CGM is that the sensor measures interstitial glucose, a parameter which may lag arterial glucose, particularly in times of flux. CGMs do display critical—potentially lifesaving—directional trends in glucose and may alert patients of impending hypo- and hyperglycemic events, but are not yet approved by the FDA for the purpose of insulin dosing by the patient, much less by an automated algorithm.

Hypoglycemia remains a major cause of morbidity and mortality in patients with type 1 diabetes (2). The literature documents that physical exertion may be a significant factor in the development of dangerous episodes of hypoglycemia (3). Prevention of exercise-



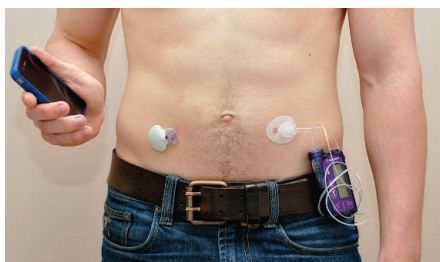
Epigenetics may
drive evolution

1082



Molecular mechanisms
meet evolution

1088



Technology in progress. The artificial pancreas has yet to be perfected.

induced hypoglycemia in a closed-loop system may require additional sensors that assess physical activity and algorithms that reduce or suspend insulin delivery during periods of high exertion.

As we strive for the perfect closed-loop pancreas system, we should not forgo the near-term opportunity to develop remotely monitored, patient-controlled systems with real-time connectivity to a central monitoring station. Such systems would over time include the use of automated algorithms, but in the near term can alert qualified health care personnel of medically actionable conditions. Monitoring station personnel could relay over-the-air modifications to the insulin pump, disable the pump if needed, and in an emergency, geolocate the patient and dispatch emergency assistance. Compared to going to sleep or exercising with an unmonitored pump, this provides the potential for increased safety. This level of telemetry and assistance is becoming commonplace in modern automobiles through telemetry systems such as OnStar® and in increasingly intelligent home-monitoring systems for burglary, fire, flood, and other hazards. Since type 1 diabetics are well known to suffer multiple hypoglycemic events per year, at an estimated average cost of \$3200 per person-year for attendant hospitalization (4), there is a plausible economic as well as medical argument for such monitoring. All that is required is the incorporation of a cellular radio into the controller of every insulin pump. Whereas the closed-loop artificial pancreas will raise patient safety concerns that will take time to overcome, few could argue that an insulin pump wearer would not be safer today with

the addition of remote telemetry and an added safety net. Why not give Americans with diabetes the same level of additional safety that we accord their automobiles and homes?

JONATHAN C. JAVITT

Johns Hopkins University, Bethesda, MD 20814, USA.
E-mail: jjavitt@telcare.com

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Supporting Mavericks

P. A. SHARP AND A. I. LESHNER ("MEETING global challenges," Editorial, 7 February, p. 579) correctly draw attention to the need for scientists and engineers to engage more meaningfully with business, cultural, and political leaders if we are to meet our most

urgent global challenges. They also stress the importance of research and teaching institutions providing appropriate training and incentives to encourage these behaviors.

But there is another dimension. A study of 36 individuals who have successfully tackled many of the world's most pressing economic, social, and environmental challenges (1) shows that most of them don't wait to reach consensus. They push on, regardless.

Consider the persistence of Nobel Laureate Barry Marshall, who overturned conventional wisdom on the genesis of gastric ulcers despite the entrenched opposition of grant agencies and the medical establishment; the self-belief that enabled environmental solutions pioneer Olivia Lum, abandoned as an infant and brought up in a palm hut without running water, to build a water purification industry that now spans the planet; and the commitment that enabled U.S. gynecologist Mitch Besser to reduce mother-to-child transmission of HIV AIDS in South Africa from 40% to 4% over the space of 10 years.

Certainly, we need to encourage collaboration in the ways identified by Sharp and Leshner. But we also need to find ways to support those with the courage to go it alone, regardless of the odds.

PETER ANDREWS

Queensland University of Technology, Brisbane, QLD, Australia. E-mail: peter.andrews@qut.edu.au

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CORRECTIONS AND CLARIFICATIONS

News Focus: "Eavesdropping on ecosystems" by K. Servick (21 February, p. 834). On p. 836, Amandine Gasc's name was misspelled in the photo credit. In a caption on the same page, French Guiana was misidentified as Guinea. The HTML and PDF versions online have been corrected.

News of the Week: "Young editors ask neuroscientists: 'Why should I care?'" by E. Underwood (24 January, p. 355). The article stated that the Nature Publishing Group has launched an online research journal geared toward young adults called *Frontiers for Young Minds*. In fact, the open access publisher *Frontiers*, part of Nature Publishing Group, launched the journal. The HTML and PDF versions online have been corrected.

News & Analysis: "Industry lobbying derails trawling ban in Europe" by T. Rabesandratana (1 November 2013, p. 544). The article incorrectly stated that the European Parliament plenary cannot reinstate the full ban on deep-sea trawling and bottom-set gillnetting proposed by the European Commission. The plenary could reconsider a full ban after the fisheries committee rejected it. The HTML and PDF versions online have been corrected.

Reports: "Stress in puberty unmasks latent neuropathological consequences of prenatal immune activation in mice" by S. Giovanoli *et al.* (1 March 2013, p. 1095). Joram Feldon, of the Laboratory of Neurobiology, ETH Zurich, 8092 Zürich, Switzerland, should be added to the authors before Manfred Schedlowski. Affiliations should be renumbered accordingly. J. Feldon should be removed from the acknowledgments. The HTML and PDF versions online have been corrected.

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

ENVIRONMENT

Culturing the Forest

Tobias Plieninger

Discourse about the state of forests around the globe has been dominated by two powerful conventional narratives: The first holds that tropical and subtropical forests are in a general decline, undergoing both degradation and deforestation. The second sees most forests as having been wilderness, devoid of humans, before European colonization. All kinds of human impact on these “pristine” forests will necessarily lead to their loss, with consequences on biodiversity, climate, and other life-supporting systems. This leads to an unavoidable trade-off between the use of forests for the needs of local people and the protection of forests for the greater good. Taken together, these narratives blend into a Malthusian worldview of humans degrading forest ecosystems. This mindset has been particularly powerful among scholars in the natural sciences [e.g., (1)].

Advances in several disciplines have fundamentally challenged many of these assumptions and drawn a more comprehensive picture of forest trajectories and the nexus between forests and people. For example, remote-sensing studies provide evidence of substantial resurgence of forests and woodlands in countries as different as El Salvador, Chile, China, and Vietnam (2). In contrast to classical assumptions of a “forest transition” (a shift from deforestation to net gains in forest area, often associated with economic development), recent forest and woodland expansion is not necessarily coupled to population declines but has also been recorded in densely populated areas (3). Long-term historical studies of ecosystems demonstrate that many remote and presumably “untouched” forests were actually heavily domesticated and intensely used and

managed landscapes, for example, in the Amazon Basin during the pre-1492 era (4). Ecological theory suggests that conservationists may focus too much on setting up wilderness areas while neglecting the need to construct a high-quality matrix of production landscapes used for agriculture and forestry (5). According to recent thinking in the land-use sciences, sustainable intensification of forestry and agriculture strongly depends on the integration of commodity production, biodiversity, and rural livelihoods at landscape scales (6).

By assembling perspectives of leading scholars from the environmental sciences, geography, anthropology, political science, history and other disciplines, *The Social Lives of Forests* builds a multifaceted view of forests as social-ecological systems. Editors Susanna B. Hecht (University of California, Los Angeles), Kathleen D. Morrison (University of Chicago), and Christine Padoch (Center for International Forestry Research, Indonesia) are well known for advocating interdisciplinary approaches to natural resources management. They have organized the volume's 28 chapters into five sections that capture important dimensions of the human-forest relationship: conceptual frameworks, historical ecologies, market dynamics, institutions, and the urban matrix.

The Social Lives of Forests Past, Present, and Future of Woodland Resurgence

Susanna B. Hecht, Kathleen D. Morrison, and Christine Padoch, Eds.

University of Chicago Press,
Chicago, 2014. 507 pp. \$50, £35.
ISBN 9780226322667.



Forest harvest. In the Peruvian Amazon, the huge fruits of Brazil nut trees (*Bertholletia excelsa*) are called “cocos” for their resemblance to coconuts.

The book opens with a number of striking cases that illustrate how frameworks, narratives, and ideologies direct our understandings of people and their roles in forests along particular pathways and how they can blind us to other views. For example, the prevailing narrative of European forest conservation policies considers high levels of biodiversity a consequence of traditional land uses. In a stark contradiction, the dominating narrative about African nature reserves considers the very same type of traditional land uses detrimental to biological conservation.

In the historical ecologies section, several case studies deconstruct the myth of untouched wilderness in places such as Mesoamerica, West Africa, Borneo, and Southwest India. The subsequent section on market dynamics reveals the social, economic, and ecologic complexities of forest commodities such as teak, shea butter, tea, and rubber. The authors show how the commodity chains of such products can support (e.g., through niche marketing) or work against (e.g., in cases of timber branding) the livelihoods of people in forests. Another section sheds light on institutional politics, in particular on how forest tenure reforms have affected human communities in forest environments. The book finishes with a series of chapters on the role of forests in an age of urbanization.

I found *The Social Lives of Forests* more a reader than a comprehensive, structured synthesis. The case studies exhibit a range of interdisciplinary approaches and methods, with comparative, place-based research at the core. By stressing the linkages among ecosystems, human well-being, livelihoods, inequality, and poverty, it follows the lines of recent initiatives such as the ICSU/UNESCO Programme on Ecosystem Change and Society. Although the editors do not provide a concluding overview or summary chapter and

the diversity of perspectives may seem overwhelming, the volume offers at least three important messages: Human-forest relationships are far more complex than simplified views of people as agents of forest degradation suggest. Mediated by prevailing institutions, politics, land-use practices, and local environments, human impact can foster multiple forest values. Inhabited “secondary forest” can support biodiversity and ecosystem services of magnitudes comparable to those of the often idealized “primordial” forest. In addition, we need to expand our under-

The reviewer is at the Department of Geosciences and Natural Resource Management, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark. E-mail: tobias.plieninger@ign.ku.dk

standing of forest to include urban parks, farm trees, wooded pastures, hedgerows, and other anthropogenic woodlands. These alternatives exhibit disparate trajectories of change and provide contributions to human well-being different from those of closed forests. Third, all types of forests—even old-growth—embody legacies of human land use, which need to be acknowledged to better understand forest dynamics and to guide forest conserva-

tion efforts. *The Social Lives of Forests* offers sophisticated, positive perspectives on forests around the world. The authors' stimulating ideas address important questions of forest dynamics and management. They also apply to the creation of working landscapes that offer space for people and nature everywhere.

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NATURAL DISASTERS

More Common Than We Think

Donald Turcotte

A large fraction of Earth's population can be adversely affected by earthquakes, volcanic eruptions, tsunamis, tornadoes, hurricanes, and floods. Many people fear the consequences of natural disasters, but few have even the most primitive appreciation of the risks. A student taking one of our natural hazards courses this year came up to the professor after a lecture and said, "We live on the Hayward fault; I didn't know we are at risk."

Almost every university offers a variety of courses on natural hazards, and professors have a wide selection of excellent textbooks from which to choose. However, their contents and costs make these accounts inappropriate for the general public. In *The Dynamics of Disaster*, Susan Kieffer addresses this void. Kieffer (a geophysicist retired from the University of Illinois) offers a fast-moving, interesting bedtime read. She imparts a range of knowledge of the risks of natural hazards in a relatively painless way that educates but also entertains. I found her inclusion of personal experiences (e.g., with earthquakes in California, tornadoes in Illinois, and floods in Australia) particularly appealing.

In the introductory chapters, Kieffer sketches a framework for understanding those disasters "caused by the ongoing geological processes on our planet." She notes that these events "result from changes of state in a system through modifications of its materials, energy, or both." After providing a short overview of the dynamics of disasters, she embarks on a

global field trip that samples their diversity.

For example, the chapter "When Terra Isn't Firma" comprises a series of short discussions on various aspects of earthquakes. Kieffer summarizes the consequences of a few major earthquakes, such as Sumatra (2004) and Japan (2011). She relates the vastly different death tolls in the magnitude-seven Haiti (2011) and New Zealand (2011) quakes to building standards. The chapter puts particular emphasis on the risks of liquefaction, and it also describes the poorly understood phenomenon of "earthquake lights."

The author covers tsunamis and ocean waves in a similar way. She describes the nature and consequences of run-up for major tsunamis. These rarely come ashore as "the spectacular wall of water ... depicted in some movies." Rather they seem like cycles of high and low tides compressed within an interval of tens of minutes. Examples of rogue waves

(single isolated gigantic waves or sets of several huge waves) provide an entertaining contrast with tsunamis (which are tiny on the open ocean). In a relatively well-documented case from World War II, RMS *Queen Mary* and the 16,000

troops aboard encountered a 90-foot-high wave in the North Atlantic that tipped the ship to a 52° list (3° short of the design limit for capsizing).

The inviting presentation of landslides includes examples of occurrences large and small, some historical and some from the geological record. (Among the latter: Fifty million years ago, at Heart Mountain, Wyo-



Aftermath. Grandmother and granddaughter searching through the rubble of their home (Miyagi Prefecture, Japan), which was destroyed in the Tohoku tsunami (11 March 2011).

ming, a sheet of rock flowed more than 50 km and covered 3300 km² in an event Kieffer estimates lasted less than an hour.) In a similarly broad and interesting treatment of atmospheric phenomena, Kieffer discusses disasters such as tornado outbreaks and severe hurricanes. She provides relatively painless introductions to Coriolis "forces" and Hadley cells as well as an engaging account of atmospheric vortices ("rotors").

The book concludes with considerations of how the public can be informed about and protected from natural disasters. Here Kieffer notes the chilling implications of the convictions of Italian seismologists for their alleged failure to warn the public of seismic risks prior to the 2009 L'Aquila earthquake.

Although some specialists may find the discussions superficial, it is hard to delve into details when considering such a broad topic in a restricted number of pages. The black-and-white illustrations may represent another compromise: while not particularly attractive, they help keep the book's price relatively low. In general, I found *The Dynamics of Disaster* a good read and strongly recommend it.

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The Dynamics of Disaster

by Susan W. Kieffer

Norton, New York, 2013.
331 pp. \$25.95, C\$27.50.
ISBN 9780393080957.

The reviewer is at the Department of Earth and Planetary Sciences, University of California, Davis, CA 95616, USA.
E-mail: dlturcotte@ucdavis.edu

CONSERVATION

Why Should We Care About Temporary Waterways?

V. Acuña,¹ T. Datry,² J. Marshall,^{3,4} D. Barceló,^{1,5} C. N. Dahm,⁶ A. Ginebreda,⁵ G. McGregor,³ S. Sabater,^{1,7} K. Tockner,^{8,9} M. A. Palmer^{10,11*}

A proposed ruling by the U.S. Environmental Protection Agency (EPA), aimed at clarifying which bodies of water that flow intermittently are protected under law (1), has provoked conflict between developers and environmental advocates. Some argue that temporary streams and rivers, defined as waterways that cease to flow at some points in space and time along their course (see the figure, left) (Fig. 1) (2), are

Failure to recognize, understand, and manage temporary waterways leads to serious degradation of aquatic ecosystems accompanied by negative impacts to the societies that depend upon them.

Current Conservation Status

Traditional flow-gauging systems have vastly underestimated the number of intermittently flowing streams and rivers in most regions,

Intermittently flowing streams and rivers should be recognized, afforded protection, and better managed.

and services. They are critical conduits for water, energy, material, and organisms even when surface water is not present (10). Shallow subsurface flows may connect dry parts of a stream or river to downstream sections that have permanent flows, are often critical for the supply of water in permanent downstream parts of a river basin, and support diverse hyporheic biota (11).

Temporary waterways are also important conduits for lateral exchanges, as they



essential to the integrity of entire river networks. Others argue that full protection will be too costly. Similar concerns extend far beyond the United States. Debate over how to treat temporary waterways in water-policy frameworks is ongoing (3), particularly because some large permanent rivers are shifting to temporary because of climate change and extraction of water (4). Even without human-induced changes, flow intermittency is part of the natural hydrology for streams and rivers globally.

We stress here the importance of policies to protect intermittently flowing streams and rivers and outline information needs that are critical to implementation of those policies.

and digital hydrological data sets used widely by water resource managers are thus unrepresentative (5). With development of novel affordable sensors, advances in remote sensing, and new modeling approaches, researchers are now showing that flow intermittency is not only very common (6, 7) but makes up the majority of river networks in many regions (8). Recent work indicates that 69% of first-order streams (the smallest) below 60° latitude flow only intermittently (see the figure, right), as do even a substantial fraction (~34%) of larger, fifth-order rivers (9).

Waterways that are naturally temporary support high biodiversity and important ecosystem processes and provide valuable goods

Different seasonal flows. (Left) The Fuirosos stream (northeast Iberian Peninsula) and (right) an intermittent tributary in Parker's Creek (mid-Atlantic U.S.) during the dry and wet seasons.

move nutrients and organisms back and forth between the channel and the floodplain or riparian vegetation region because of changes in flow. These exchanges are critical to maintenance of riparian and floodplain ecosystems and, in some arid regions, support the majority of riparian vegetation (12). Riparian vegetation, as well as vegetation that grows in dry sections of temporary river channels, provides essential wildlife habitat, forage for livestock, and wood and other ecosystem services for local people (13). Several fish species maintain healthy populations in temporary waterways; some species exhibit higher survival and reach larger sizes if they use temporary streams during early life stages (14).

Temporary streams are being buried or degraded at alarming rates owing to development, mining, hydrologic alteration, and

¹Catalan Institute for Water Research, 17003 Girona, Spain. ²National Research Institute of Science and Technology for Environment and Agriculture (IRSTEA), Aquatic Environments, Ecology and Pollution (UR-MALY), 69626 Villeurbanne, France. ³Queensland Department of Science, Information Technology, Innovation and the Arts, Brisbane 4001, Australia. ⁴Australian Rivers Institute, Griffith University, Nathan 4111, Australia. ⁵Water and Soil Quality Research Group, Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research—Spanish Council for Scientific Research (IDAEA-CSIC), 08034 Barcelona, Spain. ⁶Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA. ⁷Institute of Aquatic Ecology, University of Girona, 17071 Girona, Spain. ⁸Institute of Biology, Freie Universität Berlin, 14195 Berlin, Germany. ⁹Leibniz-Institute of Freshwater Ecology and Inland Fisheries, 12587 Berlin, Germany. ¹⁰Department of Entomology, University of Maryland, College Park, MD 20742, USA. ¹¹National Socio-Environmental Synthesis Center, Annapolis, MD 21401, USA. *Corresponding author. mpalmer@umd.edu

channel modification (15). Temporary rivers are also vulnerable because they are often used as drains to dispose of mine effluent and waste water, corridors for vehicles and livestock, and quarries for sand and gravel (13). Temporary waterways are sometimes managed as if they were permanent and, thus, may be subject to flow augmentation that leads to introduction of invasive plant and animal species (16). This widespread degradation stems from lack of recognition, poor understanding, and inadequate management.

Current Management Status

The legal status of intermittently flowing streams and rivers and the extent to which they are incorporated into policy, management, and regulatory decisions vary widely depending on how temporary waters are defined by the authorities, as well as what kinds of protection are given to temporary waterways. Even where flow intermittency is prevalent, temporary waterways may not be legally recognized as part of the river network. For example, navigable streams or tributaries in the United States are considered “jurisdictional waters” and are thus protected from filling or direct polluting. In contrast, the jurisdictional status of tributaries that do not flow continuously is determined on a case-by-case basis (17); depending on the outcome, a temporary water body may or may not be protected (table S1). This results in costly delays in regulatory decisions and confusion among landowners and natural resource managers and regulators. A proposed EPA rule that would remedy this and provide greater protection for most temporary waterways is pending public release (1).

In the European Union (EU), a temporary stream or river may or may not be considered a water body (and therefore may or may not be protected) depending on the “typology” or water body classification method adopted in a particular region (table S1) (18). Under the EU Water Framework Directive (WFD) (19), each river basin district has the authority to select one of two methods for classifying a waterway. The method generally preferred allows flexibility in what criteria other than watershed size are used in the classification process. The different criteria used in each river basin district have fostered a patchy implementation of the WFD, which has resulted in recognition of temporary waterways in few river basin districts in the EU (table S2) and in divergent outcomes.

In contrast, federal and state legal definitions in many parts of Australia explicitly include temporary streams and rivers as watercourses (table S1) (20) and thus include them in management plans that may afford

protection. For example, there is a focus on the provision of flows to protect environmental values of temporary waterways in the state of Queensland, such as by setting thresholds for the maximum duration of no-flow spells to protect the persistence of drought refuge habitats or to prevent loss of condition of river-dependent vegetation from water stress.

Policy Supported by Science

For policies to be consistent with current science, naturally temporary waterways should be legally defined as part of the river network if (i) they flow at some times and this flow connects them to a river network or (ii) they are habitat for obligate aquatic organisms or terrestrial organisms unique to dry river beds. Because temporary waterways exchange water and material with the riparia and floodplains via subsurface or intermittent surface flows, for policies to be consistent with current science, these lateral aquatic ecosystems must also be considered in management of temporary waterways.

To implement these policies, we need improved mapping of temporary waterways. In the field, they can be identified by the presence of definable channel banks or evidence of linear water flow, such as fluvially sorted bed sediments or deposits of transported organic matter. In lowland areas with poorly drained soils, channels may appear more like wetlands at points along their course because of subsurface water seepage above or below channel initiation (21). New methods to measure and predict flow intermittency are emerging (6, 22, 23). Development and refinement of biological indicators to assess and monitor the ecological status of temporary waterways, a process ongoing in some regions, will be advantageous in promoting management under new policies (11, 17).

Temporary waterways are critical hydrologically, ecologically, and socially, and their number is expected to increase in many regions over the next several decades. Policies to protect them must recognize that flow intermittency per se is not necessarily a stressor but a natural component of the flow regime of many waterways. Although economic implications of implementing new policies are likely to be debated, an economic analysis completed by the U.S. EPA concluded that costs may be minimal or offset by positive economic benefits (24). Particularly in regions where societies intimately depend on temporary waterways (e.g., arid and semi-arid regions), further degradation of water resource quality and quantity generates critical problems, with mitigation costs far exceeding those required today to halt deg-

radation with improved waterway management. Not only would broader consideration of temporary waterways in management conserve the direct values of these systems without necessarily imposing additional costs, it would also generate indirect benefits such as reducing the costs to society of flooding.

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The Secret Garden—Epigenetic Alleles Underlie Complex Traits

Robert J. Schmitz

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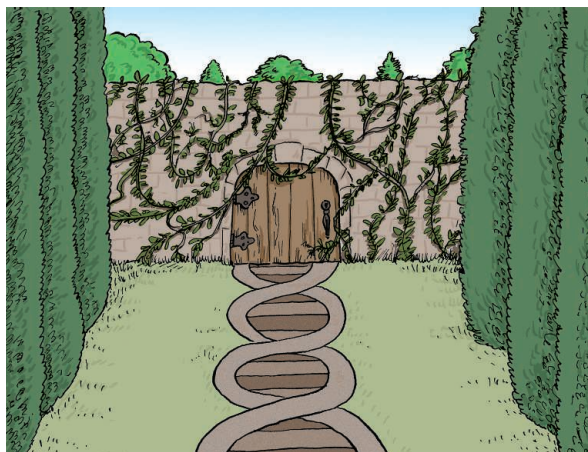
The Secret Garden—Epigenetic Alleles Underlie Complex Traits

Robert J. Schmitz

Numerous studies are investigating the basis of complex traits in a wide range of species (1). However, largely absent are efforts aimed at examining the possible contribution of natural epigenetic variation to heritable phenotypic diversity. Epigenetics is the study of heritable phenotypic variation that is not due to changes in the DNA sequence. Epigenetic variation is often overlooked because most populations used to analyze the basis of complex traits contain abundant DNA sequence variation—the major driver of phenotypic variation—and disentangling epigenetic variants from these sequence variants is a challenging task (2). On page 1145 of this issue, Cortijo *et al.* (3) demonstrate that epialleles (epigenetic alleles; alleles with the same DNA sequence but different DNA methylation patterns) are associated with heritable variation for two complex traits in the plant *Arabidopsis thaliana*. These results provide strong evidence that epialleles contribute to the heritability of complex traits and therefore provide a substrate on which Darwinian evolution may act.

Cortijo *et al.* analyzed a unique population of experimentally generated epigenetic recombinant inbred lines (epiRILs) derived from a cross between a mutant defective in DNA methylation, *ddm1* (*decreased in dna methylation 1*) (4), and its respective wild-type plant. In contrast to the widespread, albeit incomplete (5), erasure and reprogramming that happens in animals, DNA methylation is inherited across generations in plant genomes. Thus, mosaic epigenomes that are homozygous for methylation states originating from either the wild-type parent or the mutant parent could be generated: Only plants containing the wild-type *DDM1* allele were retained for further analysis (6).

Previous analyses of epiRIL populations have revealed widespread morphological variation for traits such as plant height, biomass, fruit size, number of fruits, time to flowering, germination rates, and response



to bacterial infection (6–8). Furthermore, the heritability associated with the phenotypic variance of these traits is comparable to the heritability estimates found in natural accessions of *A. thaliana* (7), suggesting that perturbation of DNA methylomes can generate heritable phenotypic diversity similar to that observed in the wild.

Whether perturbation-induced epiallelic states [observed as differentially methylated regions (DMRs)] in the epiRILs are stable enough to become targets of either artificial or natural selection has been unclear. The authors previously used the methylation states of DMRs that segregate in a Mendelian manner as physical markers to generate a genetic map (9). That such a map can even be made indicates that many of the newly induced methylation states are stable, at least over the number of generations used to create the epiRIL population. Cortijo *et al.* successfully used this genetic map for linkage mapping of two highly heritable complex traits: time to flowering (FT) and primary root length (RL). The linkage analysis revealed three quantitative trait loci (QTLs) for FT and three different QTLs for RL, and the combined additive effects of these QTLs explained large proportions of total phenotypic variation associated with either FT or RL. Therefore, this study clearly establishes that many of the epialleles derived from the creation of the epiRIL population are stable over at least eight generations and that these epialleles affect observed phenotypic variation. Identification of the causal epiallelic variants underlying each QTL is the

In the plant *Arabidopsis thaliana*, epigenetic variation, like genetic change, is potentially a substrate for Darwinian evolution.

next major challenge to demonstrate that epialleles contribute to the phenotypic variation observed in the epiRIL population.

Cortijo *et al.* relied on experimentally induced epiallele formation in artificial populations. Presently, this is the only method capable of disentangling the link between natural genetic and epigenetic variation. It raises the question, however, of whether these results are applicable to populations of *A. thaliana* accessions found in the wild. To address this limitation, Cortijo *et al.* compared the DMRs present in the epiRIL population with DMRs identified from whole-genome bisulfite sequencing of 138 natural accessions of *A. thaliana* (10) and discovered that ~30% of the DMRs in their data set are also present in the natural accessions. This overlap between experimentally induced and naturally occurring epialleles suggests that these loci are highly relevant and could contribute to at least some proportion of the often discussed “missing heritability” of complex traits (11, 12).

Cortijo *et al.* also uncovered a sizable number of allelic variants in natural populations that are silenced by DNA methylation. The method used to create the epiRIL population led to the reactivation of some of these alleles, which resulted in heritable phenotypic variation. The appreciable overlap between experimentally induced and naturally occurring epialleles could result from natural loss-of-function mutations of *ddm1* in the wild. There is also evidence in *A. thaliana* for spontaneous epiallele formation between generations (13, 14), some of which are recurrent, in the absence of *ddm1* mutations.

The detected epialleles from all of these populations, whether experimental or natural, arose in the absence of experimental environmental perturbation. The possibility of environmentally induced epiallele formation and its role in local adaptation to changing environments has generated considerable interest, but there is currently limited evidence to support the existence of these environmentally induced transgenerationally stable epialleles. An important next step will be to determine if the environment can

affect the rate at which these spontaneous epialleles appear.

Cortijo *et al.* demonstrate that heritable phenotypic variation can be achieved by perturbing DNA methylomes and reactivating silenced DNA sequences. The implications for crop systems are exciting, suggesting that heritable phenotypic variation can be rapidly generated that is linked with previously unstudied natural genetic variants. Developing techniques to precisely engineer DNA methylation sites without affecting the underlying DNA sequence will allow testing of

candidate epigenetic QTLs and examination of the potential to select for previously epigenetically silenced alleles in a wide range of plant populations. Future studies will determine the potential of these epialleles to rapidly generate heritable phenotypic variation upon which natural selection can act to alter the evolutionary trajectory of a species.

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10.1126/science.1251864

CHEMISTRY

CO Meets CO, One at a Time

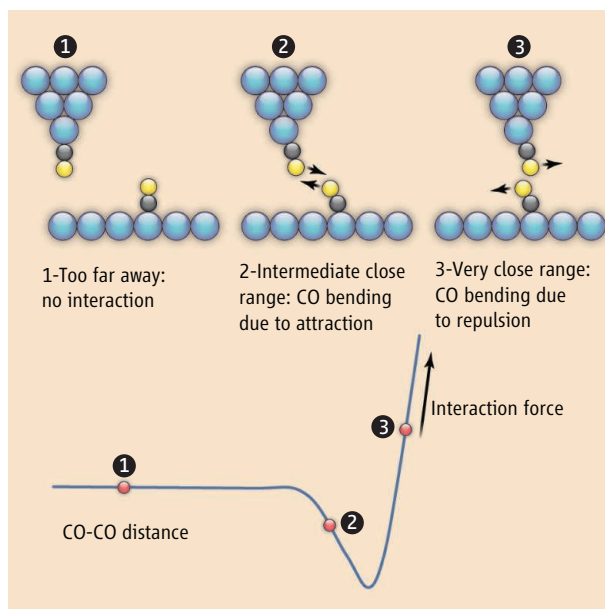
Miquel Salmeron

In his 1959 lecture, “There is plenty of room at the bottom” (1), Richard Feynman challenged scientists to build microscopes that could be used to manipulate atoms one by one. Twenty-five years later, the scanning tunneling microscope (STM) was invented (2), enabling individual atoms to be dragged to specific locations in a smooth surface (3). The atomic force microscope (AFM) (4) allowed even more sophisticated manipulations of atoms and molecules. At first sight, the importance of these feats may appear to be of an academic nature, but the wider implications in nanotechnology, which aims to manipulate matter at the atomic level to produce new materials, soon became clear. On page 1120 of this issue, Weymouth *et al.* (5) measure the forces between two single CO molecules, an example of the type of fundamental understanding that can be obtained with atomic force microscopy.

In an AFM, a sharp tip is mounted on a vibrating cantilever like that of the tuning fork used in the humble wrist watch. When the AFM is operated in noncontact mode (in which the sharp tip does not directly touch the surface), the last atom in its tip feels the tiny forces associated with attraction to and repulsion from other nearby atoms or molecules. These forces are the same as those between two atoms that are in the process of forming a chemical bond; their strength decays exponentially at distances comparable to the size of the atom.

With the noncontact AFM, one can obtain images of individual atoms and of atoms

within molecules with picometer resolution (10^{-12} m). It can also distinguish the component atoms inside the molecules by mapping the electron clouds of the chemical bonds, as shown recently in detailed images of pentacene, a planar molecule with five benzene-like rings (6). In this work, Gross *et al.* followed Eigler’s pioneering work (7) by attaching an atom or molecule to the end of the tip; this atom or molecule is then used to image the surface. This tip engineering removes the uncertainty of the atomic structure of the tip apex, a problem that plagues more standard STM and AFM imaging.



Close approach. Weymouth *et al.* attached a CO molecule to the AFM tip and then measured the changes in interaction energy as the tip came near a surface-bound CO molecule.

An atomic force microscope is used to measure the molecular forces between two carbon monoxide (CO) molecules.

The high spatial resolution achieved in noncontact AFM results from the exquisite sensitivity of the vibration frequency of the quartz tuning fork, the same reason that quartz watches keep time so accurately. Tiny changes in this frequency due to atom-atom interactions can then be used to obtain atomically resolved images when the tip is rastered over a surface. More important, the measured frequency shifts can be used for a quantitative determination of the forces at play.

Weymouth *et al.* do precisely this. They measure the shifts in the tuning-fork frequency when a CO molecule attached to the tip end is brought close to another CO molecule adsorbed on the surface of a copper crystal. As the oxygen atoms of the molecules approach each other, they first attract and later repel when their electron clouds overlap too much (see the figure). From the frequency shifts, the authors obtain a map of interaction energy between the oxygen atoms in the molecules. The departure of the repulsive part of the force from the expected curve shows that at close contacts, the molecules bend to avoid each other (see the figure).

To investigate the stiffness of this bending motion in detail, the authors next measured the frequency shift when the tuning fork and its



Water Loss from the Great Lakes
Andrew D. Gronewold and Craig A. Stow
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attached CO molecule oscillate horizontally while approaching another CO molecule on the surface. By doing this at different heights over the Cu surface, the authors were able to obtain a complete map of the forces and energies between the CO molecules.

As the result reported by Weymouth *et al.* show, noncontact AFM is a crucial tool in the quest to image atoms and molecules at ever higher resolution and to understand the origin of the forces that keep them together. From the measured forces, it is possible to identify the nature of the atoms (8) and to deduce their interaction energies. Furthermore, it opens the way for studying chemical reactions at the

individual atom or molecule level. For example, one could choose a particular site on the surface of a catalyst and position an atom or molecule there, using the tip as “tweezers.” By bringing another atom or molecule near to the first from various directions and at various distances and measuring the resulting forces, one could determine whether a reaction occurs and what the products are. In another scenario, the technique could be used to construct novel molecules by bringing the component atoms to the chosen locations one at a time. Such experiments are already happening in the laboratories of researchers. The possibilities are endless.

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ENVIRONMENT

Water Loss from the Great Lakes

Andrew D. Gronewold and Craig A. Stow

As marine coastal populations experience and plan for rising ocean levels (1), residents along the coasts of Earth's largest lake system are encountering the opposite problem: persistent low water levels and a receding shoreline. In January 2013, federal agencies from the United States and Canada documented the lowest water levels ever recorded on lakes Michigan and Huron (2). Only 6 years earlier, historically low water levels were recorded on Lake Superior (3), which feeds into the Lake Michigan-Huron system. These low water levels are symptoms of an imbalance in the water budget of the Great Lakes. Adapting to, and potentially mitigating, low water level conditions requires improved quantification of the factors that drive the imbalance.

Low water levels have a profound impact on the Great Lakes region and the North American economy by limiting navigability of shipping channels, reducing hydro-power capacity (e.g., at Niagara Falls, the largest electricity producer in New York State), impeding tourism and recreational activities, and increasing operational risks to industries that rely on the lakes as a source of process and cooling water. Relative to water levels on most marine coasts, annual water levels on the Great Lakes fluctuate widely. On Lake Michigan-Huron, for example, the historical range of recorded annual average water levels is close to 2 m (see the first figure). These fluctuations are mainly driven by

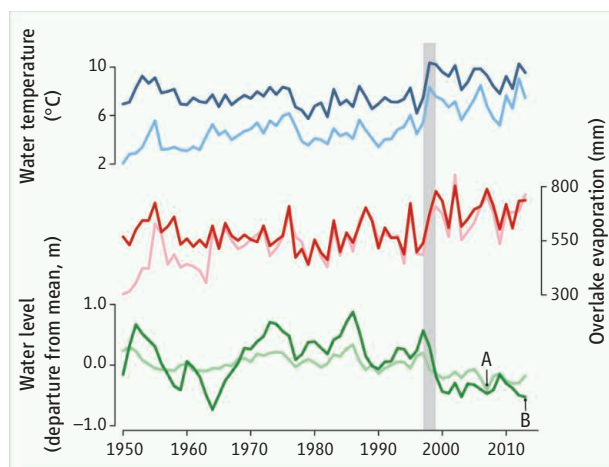
changes in regional precipitation (including both overlake precipitation and terrestrial runoff) and overlake evaporation. Water levels on Lake Michigan-Huron previously hit record lows in the mid-1960s and peaked in

Knowledge of the drivers behind recent record low water levels in the North American Great Lakes can help water resource management planning.

the mid-1980s, causing extensive erosion-related damage.

Most of the episodic changes in Great Lakes water levels over the past century are attributable to corresponding changes in annual precipitation. For example, the increases in water levels across all of the Great Lakes in the late 1960s, early 1970s, and early 1980s, as well as the water level drop in the late 1980s, are more closely linked to trends in precipitation than overlake evaporation (4). However, the large water-level drop in the late 1990s coincided with one of the strongest El Niño events on record (see the first figure) and rising surface water temperatures (~2.5°C from 1997 to 1998) on Lakes Superior and Michigan-Huron. Strong El Niño events typically lead to abnormally mild winters in the Great Lakes, and the 1997–1998 El Niño appears to show an amplification of that relationship.

Since the late 1990s, the higher surface water temperatures have persisted, promoting increased overlake evaporation,



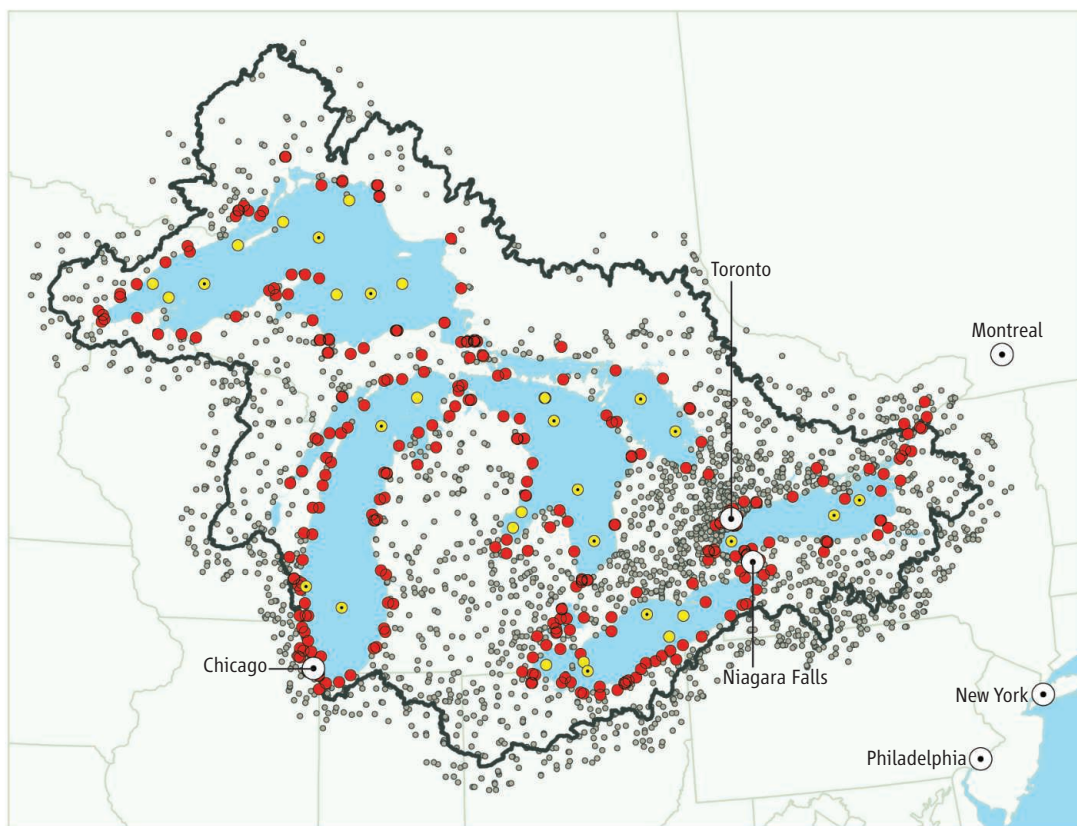
Regional climate trends. Annual climate and hydrological variables for Lake Superior (light colors) and Lake Michigan-Huron (dark colors) reflect long-term trends and abrupt shifts in surface water temperature (blue lines) and overlake evaporation (red lines). These factors have contributed to recent record low water levels on both lake systems (green lines). Water surface temperature and overlake evaporation estimates are based on computer models that assimilate measurements from a sparse network of shoreline-based stations, offshore stations (many of which are currently inactive), and seasonal offshore buoys (see second figure). Water levels are based on a comprehensive international network of shoreline monitoring stations. The vertical gray band indicates the approximate period of the 1997–1998 El Niño. Point A indicates record lows on Lake Superior for the months of August and September (both in 2007). Point B indicates record lows on Lake Michigan-Huron for the month of December (in 2012) and for all months (in January 2013). Data are from (10, 14).

National Oceanic and Atmospheric Administration (NOAA), Great Lakes Environmental Research Laboratory (GLERL), Ann Arbor, MI, USA. E-mail: drew.gronewold@noaa.gov

decreased winter ice-cover, and a period of sustained low water levels. At the same time, annual precipitation throughout the Great Lakes basin has, relative to overlake evaporation, shown minimal net change, although extreme dry conditions in 2012 combined with the already below-average water levels to produce the record seasonal lows of late 2012 and early 2013.

The low levels have catalyzed demands to regulate outflows from Lake Michigan-Huron (5); however, the demands are often aimed at reversing reductions associated with historical dredging (6) and interbasin diversions (7). Differentiating potential justifications for raising water levels on Lake Michigan-Huron is critical; compensating for historical dredging alone would do little to alleviate low water-level problems if declines continue due to climate change. Water resource management planning decisions thus hinge critically on determining the extent to which the water-level drop in the late 1990s was a state shift resulting from a strong climate perturbation (the 1997–1998 El Niño); part of a progressive decline resulting from global climate change (8, 9); or a consequence of engineering modifications to the hydrologic system, including historical channel dredging and regulation of Lake Superior outflows. Resolving these questions, however, is not straightforward.

For example, historical estimates of overlake evaporation and lake-wide surface water temperature in the Great Lakes (see the first figure) are based on computer models (10) that assimilate intermittent measurements from a small set of coastal and offshore meteorological monitoring stations (see the second figure). The coarse resolution of the monitoring network over the lakes themselves, coupled with spatial heterogeneity in regional meteorological and climatological conditions (particularly between the land and lake surface), presents a potential source of bias and uncertainty in the historical estimates (11).



Sparse monitoring networks. The Great Lakes basin (black outline) long-term hydrometeorological monitoring network shown here includes shoreline (red) and offshore (yellow) stations that, for at least 1 year in the historical record, reported measurements used in model simulations of overlake evaporation and lake surface water temperature. Of these shoreline and offshore stations, about half reported data for 10 years or less; many (yellow with concentric black dots) are seasonal buoys that are only deployed between May and November. The remaining stations (dark gray) are either too far from the lake shoreline to be potentially useful in overlake simulations or only monitor variables (such as precipitation) not used in overlake evaporation and surface water temperature simulations. For location sources, see (15).

These uncertainties complicate planning decisions but should be greatly reduced by a new and expanding network of year-round offshore monitoring stations. Within the past 5 years, for example, six fixed eddy-flux towers have been installed on remote lighthouses across the Great Lakes (12). Furthermore, scientists are exploring buoy-based sensors that could increase the spatial resolution of evaporation-related measurements substantially (13). Findings from these monitoring platforms may help to improve understanding of the water budget and of the drivers of water loss from Lakes Superior and Michigan-Huron.

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Feedback of the Magnetosphere

Joseph E. Borovsky
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GEOPHYSICS

Feedback of the Magnetosphere

Joseph E. Borovsky

For half a century, it has been known that the solar wind drives geomagnetic activity in Earth's magnetosphere. Changes in the solar-wind plasma produce perturbations in ground-based magnetometers and can prompt changes in Earth's auroral activity. When the coupling of the solar wind to the magnetosphere becomes particularly strong, a geomagnetic storm can occur, which may result in interference with communications, damage to spacecraft, and risk to high-latitude power grids (1). On page 1122 of this issue, Walsh *et al.* (2) report satellite measurements that quantify important modifications to this coupling between the solar wind and Earth's magnetosphere.

Magnetic-field-line reconnection (3) is the fundamental mechanism that enables the coupling of the supersonic solar-wind plasma to Earth's magnetosphere. At the sunward boundary of the magnetosphere, magnetic-field-line reconnection connects the magnetic field of the moving solar-wind plasma to the magnetic field of Earth, enabling the

flow of electrical currents and the exchange of mass, momentum, and energy between the solar wind and the magnetosphere. Whatever controls the rate of reconnection on this boundary controls the rate of coupling. Until recently, it was believed that the solar wind entirely controlled the reconnection rate.

Magnetospheric physics is evolving toward a systems-science approach to the operation of the complex magnetosphere-ionosphere system (4, 5). In this approach, the behavior of the entire interacting system is considered when studying any aspect of the system. Cold-plasma outflow from the ionosphere accumulates in the magnetosphere to high densities forming a reservoir region near Earth known as the plasmasphere; when solar-wind coupling becomes strong, some of that stored cold plasma is released and transported from the near-Earth reservoir to the sunward boundary of the magnetosphere (6, 7). Satellite imaging reveals a plume of this cold plasma draining from the plasmasphere and advecting toward the sunward boundary (see the figure). Measurements have confirmed that this plasma flows into the site of reconnection on the sunward boundary (8). This inflow of

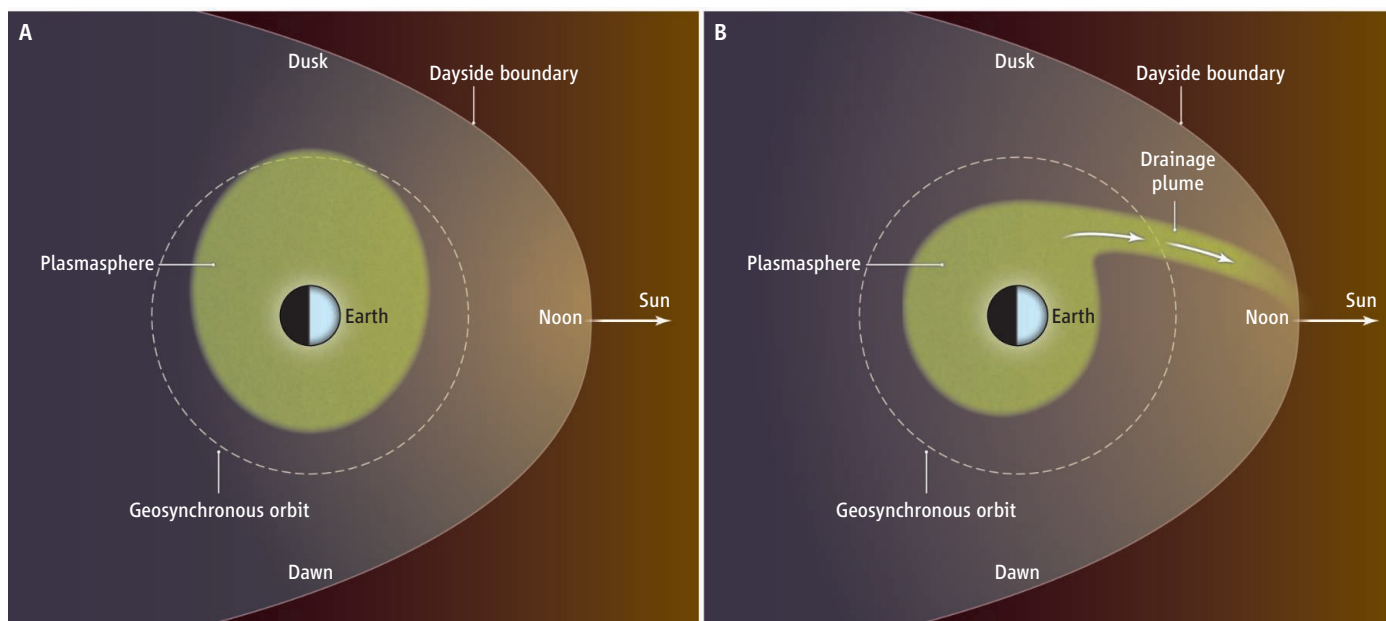
A dense cold plasma from Earth's ionosphere can counter strong solar wind.

dense plasma could mass-load the reconnection site to reduce the rate of reconnection and, hence, reduce the coupling of the solar wind to the magnetosphere (9).

The plasmasphere effect has several classic systems-science aspects (10): It represents a negative-feedback loop to the driving of the system—that is, the stronger the driving, the more rapidly plasma is fed into the reconnection site; the strength of this feedback depends on the several-day time history of the magnetosphere, i.e., on how full the plasma reservoir is; time lags are introduced into the response of the magnetospheric system, as the leading edge of dense plasma takes minutes to reach the reconnection site and then days for the reservoir to be depleted; it is a system-wide phenomenon involving the ionosphere, the near-Earth magnetosphere, the sunward boundary of the magnetosphere, and the solar wind; and it involves diverse physical processes such as ionospheric outflows, magnetospheric transport, and magnetic-field-line reconnection.

Until recently it had been thought that the motional electric field of the magnetized solar-wind plasma controlled the rate of reconnection (11). The new Cassak-Shay

Center for Space Plasma Physics, Space Science Institute, Boulder, CO 80301–2532, USA. E-mail: jborovsky@space-science.org



Pushing back. From a perspective high over the North Pole of Earth, the cold plasma in the equatorial plane of Earth's magnetosphere is sketched at two different times. (A) When solar-wind coupling is weak, the near-Earth reservoir (plasmasphere) is shown in green. (B) When coupling becomes stronger, the

plume of sunward-convecting cold plasma eroding from the reservoir is seen. The cold plasma of the plume flows to the dayside boundary of the magnetosphere, where it interferes with the reconnection process. Space-based ultraviolet images of this cold-plasma movement can be seen in Goldstein (7).

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approach (12) to calculating reconnection rates in plasmas explicitly allows for quantification of this plasmasphere effect. In the Cassak-Shay formulation, the reconnection rate is sensitive to the mass density of each of the two plasmas coming into contact—the solar wind and the magnetosphere. Previous work reported a critical statistical analysis of measurements of the mass density of this cold magnetospheric plasma entering the boundary reconnection site (13). Now, Walsh *et al.* put together observations of the complete flow of this cold plasma from the near-Earth regions to the sunward boundary of the magnetosphere and supply satellite measurements that show that the plasma indeed mass loads the rate of reconnection.

The plasmasphere effect is indicative of a new level of sophistication in the understanding of how the magnetospheric system operates. The effect can be particularly important for reducing solar-wind/magnetosphere coupling during geomagnetic storms. Instead of unchallenged solar-wind control of the rate of solar-wind/magnetosphere coupling, we see that the magnetosphere, with the help of the ionosphere, fights back.

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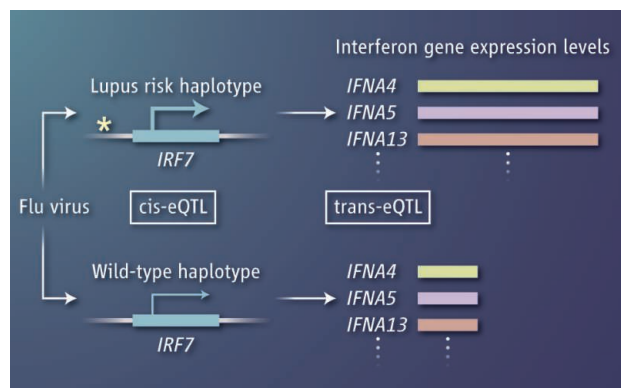
GENETICS

A Genomic Road Map for Complex Human Disease

Peter K. Gregersen

Despite the successes of genome-wide association studies (GWAS) in identifying genetic connections with human disease, it has become clear that interpreting these data requires a clear understanding of how these new risk genes are regulated. On pages 1118 and 1119 of this issue, Fairfax *et al.* (1) and Lee *et al.* (2), respectively, elucidate networks of genetic regulation in the context of the human innate immune system and show how this information can be directly applied to understanding the genetics of autoimmune disorders.

The human immune system can be broadly divided into the innate and adaptive immune systems. The latter enables the formation of immune memory through T cell responses and the production of antibodies by B cells. Although many genetic variants control these processes, the human major histocompatibility complex [(MHC); cell surface molecules that mediate interactions with immune cells] has a predominant role in determining specificity of the adaptive immune response.



Consideration of cell type- and disease-associated environmental conditions is critical to connecting specific genetic variants to immune disorders.

Variation and response. eQTL information can be integrated with GWAS information to better understand how genetic variants are linked to innate immune responses to infection. Shown is the locus of the *IRF7* gene in dendritic cells from two individuals. The top locus has a SNP (asterisk) that is associated with susceptibility to the autoimmune condition lupus. This locus is also an eQTL for the dendritic cell response (interferon production) to infection with influenza virus. Elevated production of interferon is also often observed in lupus, a disease that has been associated with abnormalities in dendritic cells (8).

By contrast, the innate immune system comprises phylogenetically older mechanisms of immediate immune defense with various cellular components, many of which interact with the adaptive immune system. Two innate immune cells, monocytes and dendritic cells, are the focus of studies by Fairfax *et al.* and Lee *et al.*, respectively.

Gene expression can be quantified according to the amount of messenger RNA (mRNA) in cells and is therefore a quantitative trait. Genetic variants that regulate mRNA expression are one form of quantitative trait locus (QTL), or eQTL. Early investigations of eQTLs often examined cell lines or peripheral blood. However, the message that emerges from Fairfax *et al.* and Lee *et al.* is that the patterns of eQTLs are highly dependent on the specific cell type examined, and

even more strikingly, on the in vitro environmental conditions to which a cell is exposed. Both groups focused on immune activators, including viruses, cytokines (interferon), and bacterial products such as lipopolysaccharide (LPS). These exposures are directly relevant to disease-related phenotypic differences among individuals.

Although this general message may be straightforward, and even expected, obtaining supporting data is not trivial; the scientific treasures are to be found in the complexity of the experimental details. There are many potential sources of variation in gene expression, and providing robust evidence for genetic variation interacting with environmental exposures requires the study of hundreds of subjects with defined genotypes, comprehensive and reproducible gene

Robert S. Boas Center for Genomics and Human Genetics, The Feinstein Institute for Medical Research, North Shore LIJ Health System, 350 Community Drive, Manhasset, NY 110430, USA. E-mail: peterg@nshs.edu



A Genomic Road Map for Complex Human Disease

Peter K. Gregersen

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<http://www.sciencemag.org/cgi/collection/immunology>

approach (12) to calculating reconnection rates in plasmas explicitly allows for quantification of this plasmasphere effect. In the Cassak-Shay formulation, the reconnection rate is sensitive to the mass density of each of the two plasmas coming into contact—the solar wind and the magnetosphere. Previous work reported a critical statistical analysis of measurements of the mass density of this cold magnetospheric plasma entering the boundary reconnection site (13). Now, Walsh *et al.* put together observations of the complete flow of this cold plasma from the near-Earth regions to the sunward boundary of the magnetosphere and supply satellite measurements that show that the plasma indeed mass loads the rate of reconnection.

The plasmasphere effect is indicative of a new level of sophistication in the understanding of how the magnetospheric system operates. The effect can be particularly important for reducing solar-wind/magnetosphere coupling during geomagnetic storms. Instead of unchallenged solar-wind control of the rate of solar-wind/magnetosphere coupling, we see that the magnetosphere, with the help of the ionosphere, fights back.

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10.1126/science.1250590

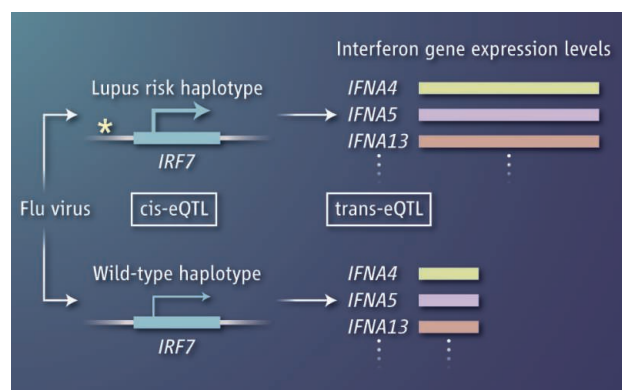
GENETICS

A Genomic Road Map for Complex Human Disease

Peter K. Gregersen

Despite the successes of genome-wide association studies (GWAS) in identifying genetic connections with human disease, it has become clear that interpreting these data requires a clear understanding of how these new risk genes are regulated. On pages 1118 and 1119 of this issue, Fairfax *et al.* (1) and Lee *et al.* (2), respectively, elucidate networks of genetic regulation in the context of the human innate immune system and show how this information can be directly applied to understanding the genetics of autoimmune disorders.

The human immune system can be broadly divided into the innate and adaptive immune systems. The latter enables the formation of immune memory through T cell responses and the production of antibodies by B cells. Although many genetic variants control these processes, the human major histocompatibility complex [(MHC); cell surface molecules that mediate interactions with immune cells] has a predominant role in determining specificity of the adaptive immune response.



Consideration of cell type- and disease-associated environmental conditions is critical to connecting specific genetic variants to immune disorders.

Variation and response. eQTL information can be integrated with GWAS information to better understand how genetic variants are linked to innate immune responses to infection. Shown is the locus of the *IRF7* gene in dendritic cells from two individuals. The top locus has a SNP (asterisk) that is associated with susceptibility to the autoimmune condition lupus. This locus is also an eQTL for the dendritic cell response (interferon production) to infection with influenza virus. Elevated production of interferon is also often observed in lupus, a disease that has been associated with abnormalities in dendritic cells (8).

By contrast, the innate immune system comprises phylogenetically older mechanisms of immediate immune defense with various cellular components, many of which interact with the adaptive immune system. Two innate immune cells, monocytes and dendritic cells, are the focus of studies by Fairfax *et al.* and Lee *et al.*, respectively.

Gene expression can be quantified according to the amount of messenger RNA (mRNA) in cells and is therefore a quantitative trait. Genetic variants that regulate mRNA expression are one form of quantitative trait locus (QTL), or eQTL. Early investigations of eQTLs often examined cell lines or peripheral blood. However, the message that emerges from Fairfax *et al.* and Lee *et al.* is that the patterns of eQTLs are highly dependent on the specific cell type examined, and

even more strikingly, on the in vitro environmental conditions to which a cell is exposed. Both groups focused on immune activators, including viruses, cytokines (interferon), and bacterial products such as lipopolysaccharide (LPS). These exposures are directly relevant to disease-related phenotypic differences among individuals.

Although this general message may be straightforward, and even expected, obtaining supporting data is not trivial; the scientific treasures are to be found in the complexity of the experimental details. There are many potential sources of variation in gene expression, and providing robust evidence for genetic variation interacting with environmental exposures requires the study of hundreds of subjects with defined genotypes, comprehensive and reproducible gene

Robert S. Boas Center for Genomics and Human Genetics, The Feinstein Institute for Medical Research, North Shore LIJ Health System, 350 Community Drive, Manhasset, NY 110430, USA. E-mail: peterg@nshs.edu

expression analysis, careful isolation of the proper cell subsets, and controlled in vitro stimulations with respect to dose and timing.

eQTLs may act in cis (regulatory variants located near the gene under regulation), or in trans (regulation by variants at great distance, including on different chromosomes). There are many examples of cis regulation, but trans regulation has been more difficult to sort out due to its complexity and the large number of potential variants to investigate. It is therefore gratifying that both studies show an integrated network of cis and trans regulation. Fairfax *et al.* identify, within the Toll-like receptor (TLR) pathway (which responds to bacterial LPS), the transcription factor interferon regulatory factor 2 (IRF2) as an eQTL in monocytes. Lee *et al.* demonstrate trans-eQTLs of IRF7 in dendritic cells under several conditions. Adding to this complexity, it is apparent that trans-eQTLs can also be time dependent, mediated indirectly through intervening signaling pathways. For example, interferon B1 (IFNB1) is among the cis-eQTLs in monocytes that is induced by LPS at 2 hours after exposure. However, at 24 hours after exposure, this cis-eQTL is no longer observed, but multiple trans-eQTLs appear in pathways downstream of IFNB1. Although Lee *et al.* restricted their full analyses of LPS-triggered responses to an early time point (5 hours after exposure), it is clear from their exploratory time-course data that similarly connected time-dependent eQTL responses are likely to exist in dendritic cells. One example of an additional mechanistic twist involves a coding-region eQTL in the gene encoding cytochrome P450 1B1 that leads to shortened protein half-life and consequent compensatory feedback on transcription of its own mRNA. Clearly, the experimental details are critical for eQTL discovery and mechanistic understanding.

Such information can be applied to understanding GWAS data. For example, the haplotype bearing the IRF7 trans-eQTL contains a single-nucleotide polymorphism that is associated with systemic lupus and is also an eQTL for induction of a robust interferon response to viral exposure (see the figure), a finding consistent with abnormal regulation of interferon in this disease (3). Similarly, Fairfax *et al.* show that an interferon-dependent eQTL in the gene encoding caspase recruitment domain-containing protein 9 (CARD9), a protein associated with the inflammatory bowel disorder Crohn's disease (4), is actually a secondary independent disease-associated variant in the *CARD9* locus. Indeed, the studies of Fairfax *et al.* and Lee *et al.* reveal a great deal of overlap between

eQTL loci and a variety of immune-related disorders. Furthermore, Lee *et al.* show that many eQTL loci have multiple variants with distinct effects, emphasizing the complexity of interpreting associations identified by GWAS, even at a single locus.

An intriguing observation by Fairfax *et al.* is that interferon acts through the MHC to induce many trans-eQTL effects in isolated monocytes. The MHC is one of the most gene-dense and highly polymorphic regions in the genome, so it can be difficult to pinpoint exactly which genetic variants are responsible for these trans-eQTL effects. The MHC is the predominant genetic locus controlling adaptive immunity and many autoimmune diseases, primarily through interactions with T cell receptors. For these eQTL effects on monocytes, however, T cells are not present in the system. Nevertheless, eQTL effects are correlated with the cis-eQTL effects on class II MHC expression, suggesting that the class II molecules themselves may be mechanistically involved. This is consistent with studies showing intracellular signaling by MHC class molecules (5),

as well as their ability to modify signaling, through TLR pathways (6).

eQTLs for gene expression that are cell type- and context-specific will be reflective of quantitative immune traits at the system and organismal levels (7, 8), which in turn constitute phenotypes that are likely to determine disease susceptibility (9). Elucidating these relationships is a major challenge, and will require extensive collaboration among clinical and basic investigators, as well as scientists with advanced computational expertise.

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10.1126/science.1251426

MEDICINE

Combating Evolution to Fight Disease

Susan M. Rosenberg¹ and Christine Queitsch²

Molecular mechanisms that generate biological diversity are rewriting ideas about how evolution proceeds, with implications for treating disease.

Molecular biology and evolutionary biology have been separate disciplines and scientific cultures: The former is mechanistic and focused on molecules; the latter is theoretical and focused on populations. However, these domains are beginning to converge in laboratories addressing molecular mechanisms that explain how evolutionary processes work, and bring these processes to bear on medical problems such as cancer and infectious disease. Each discipline can be viewed as a missing link in the other's description of biology, and in medicine.

¹Departments of Molecular and Human Genetics, Biochemistry and Molecular Biology, Molecular Virology and Microbiology, and Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, USA. ²Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA. E-mail: smr@bcm.edu

Traditional evolutionary biology began in the 1930s with the "modern synthesis," which fused Darwin's theses on phenotypic variation and selection with Mendel's concepts of genetic inheritance to explain the source of biological diversity. This synthesis predated knowledge that genes were made of DNA and of the structure of DNA and how it replicates. Thus, molecular mechanisms could not be integrated into concepts about how phenotypic variation is generated. Instead, assumptions had to be made about the origins of the variation that drives evolution. Among the cornerstone assumptions were that mutations are the sole drivers of evolution; mutations occur randomly, constantly, and gradually; and the transmission of genetic information is vertical from parent to offspring, rather than horizontal (infectious) between individuals and species (as is now apparent throughout the tree

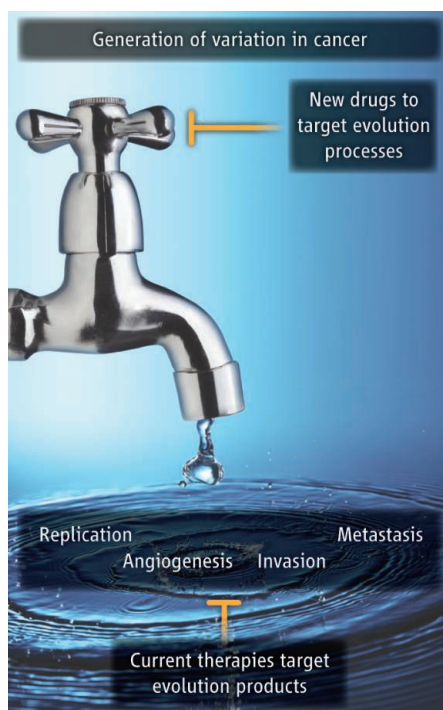
of life). But discoveries of molecular mechanisms are modifying these assumptions.

In at least two ways, heritable variation can be generated by proteins, not DNA (1). Spontaneously self-aggregating alternative conformations of some proteins—prions—can flip into their aggregated state and change a cell's phenotype in an environmentally responsive manner with no change to DNA. The change is transmissible vertically, parent to offspring cell, as well as horizontally, to other cells in which the proteins come in contact. Another mechanism involves chaperones such as heat shock protein 90 (Hsp90), proteins that massage subideal (mutant) proteins into functional conformations but abandon their regular client proteins during heat and other stresses that destabilize proteins. This causes a stress-inducible release of phenotypic diversity, which may drive evolution (with phenotypes ultimately stabilized by subsequent genetic changes). Both of these molecular mechanisms of protein-based inheritance are major departures from the modern synthesis views of solely mutation-directed variation, solely genetic inheritance, and independence of the generation of variation from environmental conditions.

Similarly, transient errors in mRNA synthesis can also cause heritable non-DNA-based phenotypic change. This is observed when low-abundance transcriptional regulators are affected by transcription errors. This disruption can cause a cell to alter its gene expression, resulting in a phenotype that may be heritable (2).

Even the assumption that mutations are random, constant, and gradual has been revised on the basis of molecular mechanisms of mutagenesis. For example, in bacteria, responses to environmental stress can activate mutagenesis mechanisms that increase mutation rate, which can potentially increase the ability of a cell to evolve, specifically when it is poorly adapted to its environment (when stressed). Most of a 93-gene network that promotes mutagenesis in *Escherichia coli* is devoted to sensing stress and activating stress responses that direct the bacterium to mutate when stressed (3). Stress responses also up-regulate mutagenesis in yeast (4) and human cancer cells (5) and underlie mutations induced by antibiotics that cause resistance to those very drugs, and others (6).

Mutations are also nonrandom in genomic space—for example, forming hot spots at DNA double-strand breaks, as demonstrated in bacteria (7) and suggested by local clusters of mutations in cancer genomes (8, 9). In cancer, the mutations are generated by cytidine deaminases that tar-



“Anti-evolvability” therapies. Multiple molecular mechanisms including mutation, protein conformational change, and epigenetic gene silencing create phenotypic variation that, with selection for the fittest cells, drives cancer initiation, progression, and resistance. Whereas conventional antiproliferative therapies target the products of this somatic cell evolution, proposed new therapies that block the evolutionary processes by which phenotypic variation is generated may be effective.

get single-stranded DNA regions (10), presumably at DNA breaks. Additionally, the structure of the human genome with regard to repetitive DNA (11) and three-dimensional structure (12, 13) predisposes certain regions to copy number variation because of recombination between repeats (11) or proximity in the nucleus of nonrepeated sequences (12, 13). The long-standing assumption of random, constant, and gradual mutagenesis is refuted by observations that mutations occur more frequently when cells are maladapted to their environments, together with the discoveries of mechanisms by which mutations are targeted to specific genomic structures. These modifications of the modern synthesis assumptions could not have been predicted or found without exploration of molecular mechanisms.

Such a fusion of molecular mechanisms with evolution is needed because cancer and infectious disease are evolutionary problems that could be attacked at the molecular level. For example, when a pathogen defeats a host, it has won an evolutionary arms race with the host immune system; cancer, too, proceeds

by the generation of phenotypic variation and selection of those cells that are most fit to propagate (see the figure). Deep understanding of the mechanisms that generate variation at the molecular level invites the possibility of fundamentally new antipathogen and anticancer therapies: ones that block the ability to evolve, instead of (or in addition to) traditional chemotherapies that kill cells or stop them from growing. For example, inhibitors of Hsp90 are succeeding as antifungal therapy adjuncts that block the development of resistance to standard drugs (14) and are in trials as chemotherapeutic agents. Similarly, the heat shock response factor that regulates Hsp90 has emerged as a promising therapeutic target in cancer (15). Conventional cancer chemotherapies target the products of tumor evolution (such as DNA replication, altered cell signaling that promotes rapid proliferation, or the release of factors by cancer cells that spur angiogenesis). “Anti-evolvability” therapies could potentially stem the torrent of generation of variation that creates all of the products of evolution, whether targetable directly by drugs or not, thereby blocking the processes of tumor or pathogen evolution (see the figure).

The evolutionary biologist Theodosius Dobzhansky famously noted that “nothing in biology makes sense except in the light of evolution,” but perhaps, too, “nothing in evolution makes sense except in the light of biology.” Although the latter might be an exaggeration, an important gap is being filled by molecular understanding of the genesis of variation that confers the ability to evolve.

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INTRODUCTION

Going from Strength to Strength

IN 1912, THE GERMAN PHYSICIST MAX VON LAUE PUBLISHED THE FIRST PAPER demonstrating x-ray diffraction from a crystal. This discovery, for which he was awarded the Nobel Prize in 1914, provided a window into the regular atomic arrangements within crystals. Today, the Cambridge Structural Database contains more than 600,000 structures of organic and organometallic molecules, many obtained through x-ray crystallography; the Protein Data Bank contains about 100,000 structures. The insights gained from these and other structural studies have revolutionized understanding of chemical and biological systems, leading to the award of 29 Nobel Prizes for scientific achievements related to, or involving the use of, crystallography.

In their Review (p. 1098), Howard and Probert highlight advances in studying single crystals of nonbiological molecules and materials. Novel approaches are helping crystallize unstable samples and mount them in the x-ray diffractometer without damaging the fragile crystals. Advanced x-ray sources allow structures to be obtained from smaller crystals and provide access to time-resolved data on chemical reactions within crystals. Crystals can now be studied at low temperatures and high pressures, further extending the range of conditions and samples that can be structurally characterized.

Garman (p. 1102) charts the history of structural biochemistry, from the initial report of x-ray diffraction from pepsin crystals to the recent characterization of the entire ribosome and of G protein-coupled receptors in different conformational states. She discusses the challenges of protein crystallization, which is increasingly automated. The vast majority of protein structures come from synchrotron beamlines, many of which now offer sample-mounting robots, microfocus beams, and the ability to collect supplementary (e.g., spectroscopic) data. Radiation damage may be overcome through the use of x-ray free-electron lasers. In a related Perspective in *Science Signaling*, Smerdon discusses the insights into the regulation of the kinase mTOR gained from protein crystallography.

Building on the success in obtaining static structures, Miller (p. 1108) discusses efforts to capture atomic motions in crystals in real time. Very bright tabletop electron sources have been used to study photoinduced phase transitions and photoinduced organic reactions. Time-resolved x-ray diffraction experiments are mainly performed at synchrotron light sources, although the development of tabletop instruments is under way. X-ray free-electron lasers offer exciting opportunities for time-resolved studies, particularly of biomolecules.

Science's News writers mark crystallography's centenary with a timeline by Sumner (p. 1092) highlighting some of the field's most celebrated discoveries and advances. Service (p. 1094) describes researchers' long quest for the elusive structures of the proteins that act as gatekeepers to cell membranes. Finally, in News Focus (p. 1072), Service reviews the Protein Structure Initiative, a major research program sponsored by the U.S. National Institutes of Health, and looks ahead to how its scheduled shutdown in 2015 could affect structural biology.

— ROBERT COONTZ, JULIA FAHRENKAMP-UPPENBRINK, MARC LAVINE, VALDA VINSON

Crystallography at 100

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Science

Dazzling History

REPORTED AND WRITTEN BY THOMAS SUMNER

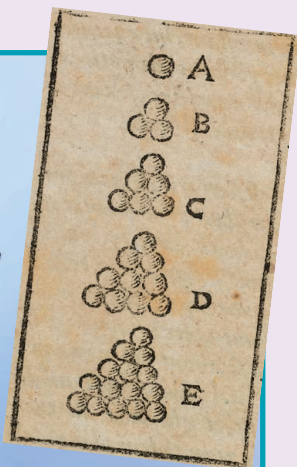
Over the past century, x-ray crystallography has transformed scientists' understanding of the structure and behavior of materials

PREHISTORY



1611

Johannes Kepler speculates that **snowflakes** are **hexagonal grids** of water particles—a hypothesis that cannot be tested for centuries to come.



1895  1901 Physics

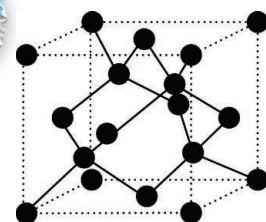
Wilhelm Röntgen produces and measures **x-rays**.

1912  1914 Physics

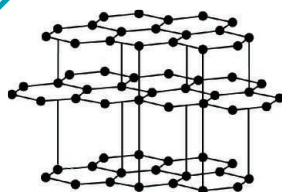
Max von Laue creates a **diffraction pattern** by firing x-rays at a crystal of copper sulfate but cannot interpret it.

1913

Braggs determine crystal structure of **diamond**.



1916 **Powder diffraction analysis** makes it possible to study small crystals.



1924

John Desmond Bernal determines structure of **graphite**.

1937  1946 Chemistry

James Sumner demonstrates that any protein can be **crystallized**.

1945  1964 Chemistry

Dorothy Hodgkin and colleagues determine structure of **penicillin**, the first complex molecule solved by x-rays.

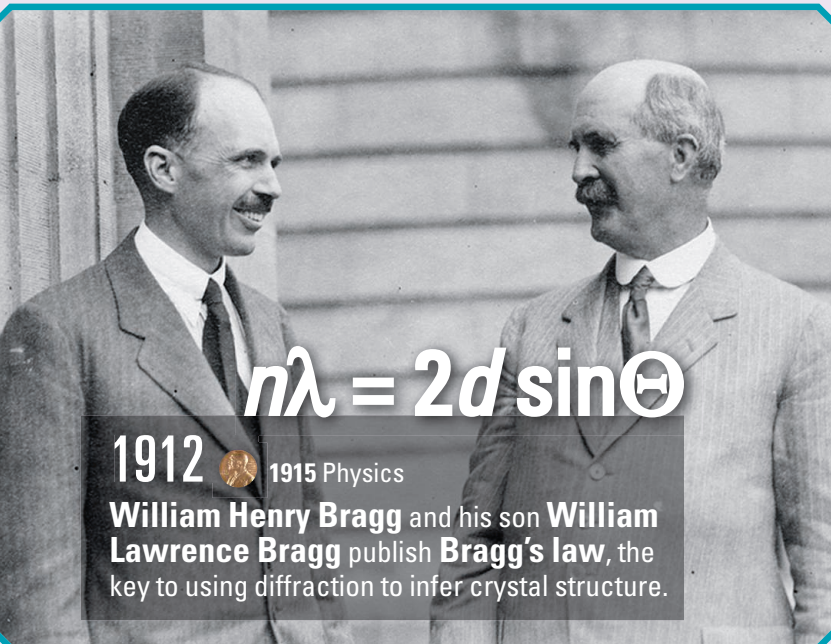
1946  1994 Physics

First **neutron diffraction** experiments; the technique provides **3D structures** and other details that x-rays cannot.

$$n\lambda = 2d \sin\Theta$$

1912  1915 Physics

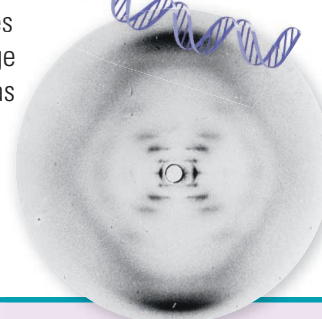
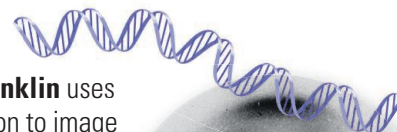
William Henry Bragg and his son **William Lawrence Bragg** publish **Bragg's law**, the key to using diffraction to infer crystal structure.



1952

Rosalind Franklin uses x-ray diffraction to image **DNA** and suggests it has a helical structure.

 1962 Physiology or Medicine
F. Crick, J. Watson, and M. Wilkins



1952

Grazing-incidence optics paves way for modern x-ray studies.

1958

 1962 Chemistry

John Kendrew and Max Perutz determine first protein structures, of **myoglobin** and **hemoglobin**.



1970

The first **synchrotron x-ray** sources open, producing brilliant x-rays for detailed crystallography research.

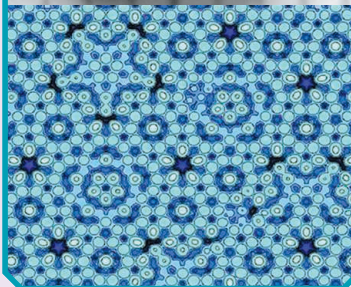
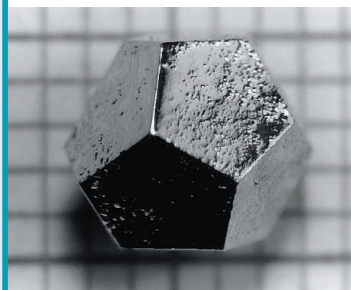
1978

Tomato bushy stunt virus is imaged—the first viral structure mapped at atomic level.

1982

 2011 Chemistry

Scientists observe first **quasicrystals**, strange materials whose atoms follow an **ordered but nonrepeating pattern**.



1984

 1988 Chemistry

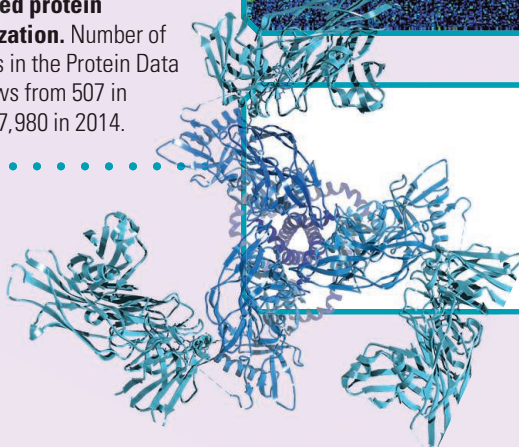
Researchers solve structure of **photosynthesis reaction site**.

1989

Time-resolved crystallography reveals action mechanisms of rapidly changing molecules.

1990s

Automated protein crystallization. Number of structures in the Protein Data Bank grows from 507 in 1990 to 97,980 in 2014.



2000

Protein Structure Initiative begins (see News Focus, in this issue).

2001

"Robotic beamlines" start to speed sample analysis at x-ray sources.

2002

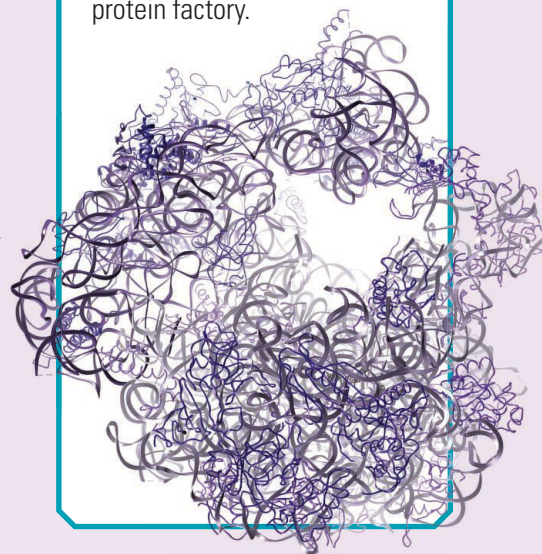
Microfluidic chips promise to boost automated protein-crystal growing.

2000



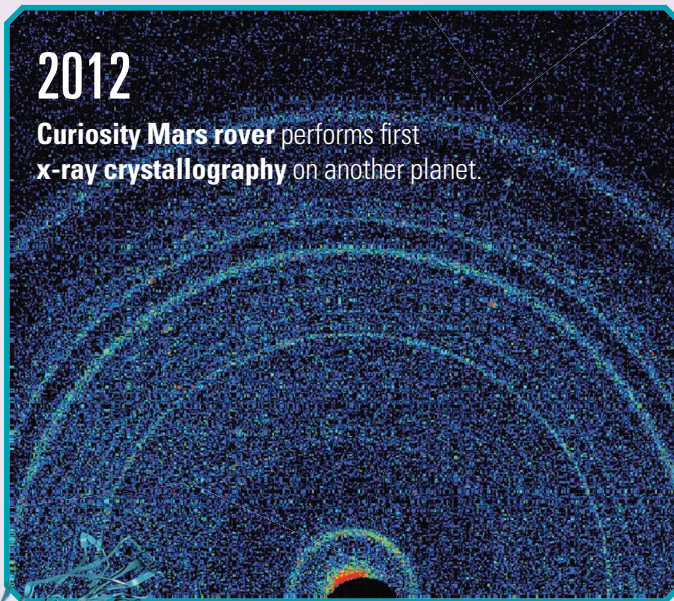
2009 Chemistry

Scientists solve structure of a **ribosome**, cells' protein factory.



2012

Curiosity Mars rover performs first **x-ray crystallography** on another planet.

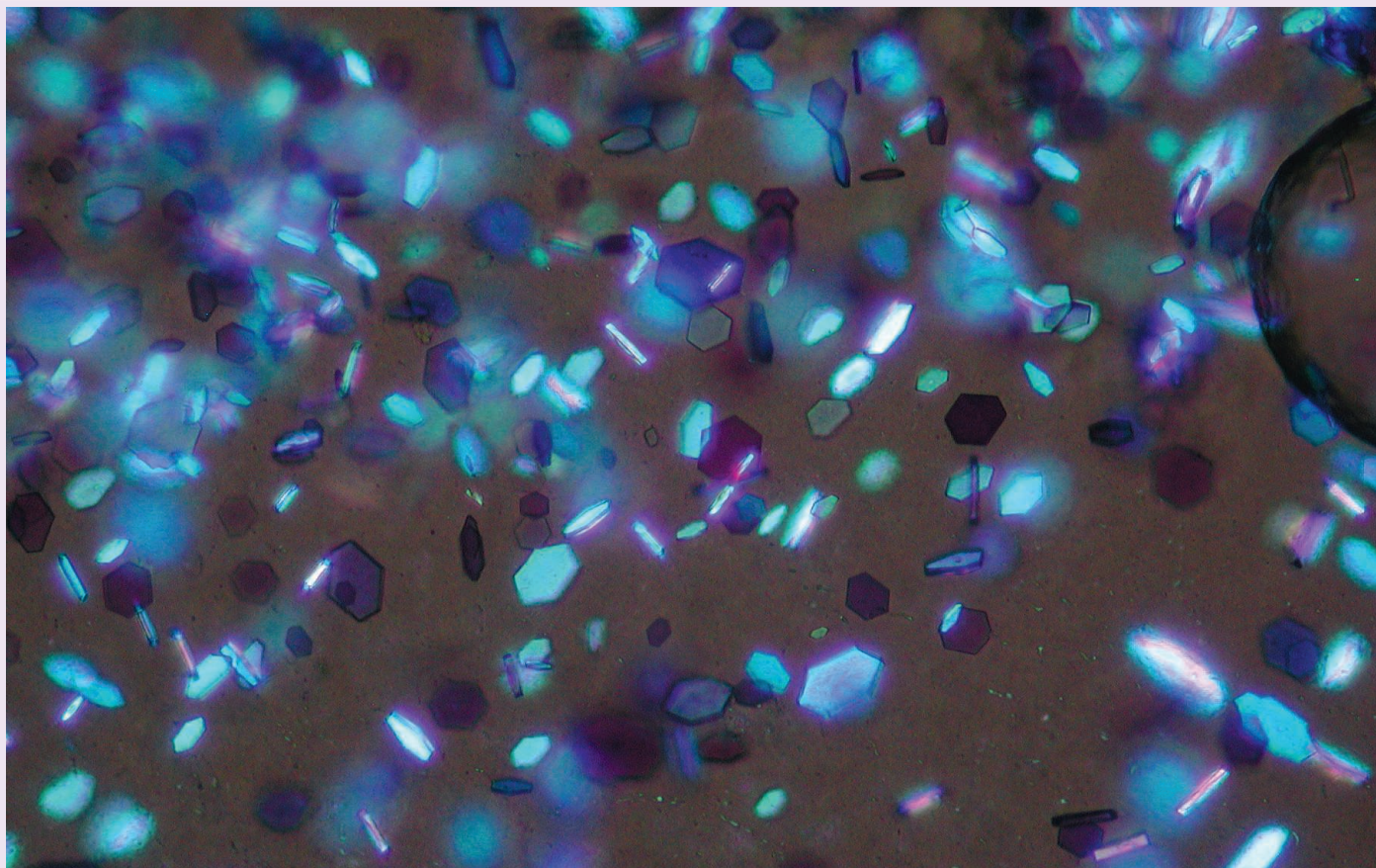


2013

Crystallography yields a detailed picture of the protein that **HIV** uses to **invade immune cells**.



Nobel Prize awarded for work



Gently Does It

A technique for crystallizing fragile biomolecules without disrupting them is helping researchers probe the structures of some of the body's most important but elusive proteins: those that usher other chemicals through the cell membrane

The tiny purple crystals, glistening within a translucent, fatty gel, signaled that Ehud Landau and Jürg Rosenbusch had made headway on one of the toughest problems in x-ray crystallography. To map a protein's atomic structure using x-rays, crystallographers have to coax its molecules to align themselves in crystals, like soldiers in perfect formation. That's difficult enough for ordinary proteins, which are complex, flexible molecules. But the membrane proteins that straddle the cell's surface and control the chemical traffic in and out are an even bigger challenge. Nestled within their normal

protective environment, membrane proteins are stable and well-behaved. But take them out to try and get them to line up, and the task is like herding cats.

Two decades ago, Landau, a chemist then at the University of Basel in Switzerland, thought the answer might lie in a curious mixture of fatlike molecules called lipids, blended with water and other compounds. The concoctions spontaneously form 3D shapes called the lipidic cubic phase (LCP), and Landau hoped they could serve as a synthetic cell membrane to keep the membrane proteins happy outside cells. He and Rosenbusch,

a structural biologist also at Basel, tested the scheme with a purple membrane protein known as bacteriorhodopsin (bR), found in halobacteria. The plan worked. The result was the 50-micron-wide bR crystals—and, in the years that followed, a mini-explosion in membrane protein crystal structures.

Membrane proteins may be the most important molecules in biology. These enzymes, receptors, channels, and transporters account for more than half of the targets for all pharmaceutical compounds on the market. And LCP has been essential for understanding them. "It's been magical

CREDIT: VADIM CHEREZOV/THE SCRIPPS RESEARCH INSTITUTE

Crystal power. Crystallizing proteins such as bacteriorhodopsin is key to solving their atomic structure.

for us,” says Wayne Hendrickson, a protein crystallographer at Columbia University, who has recently used the technique to solve two membrane protein structures.

But getting the LCP mixtures right and handling them is tricky. After their first glimpse of those purple bR crystals, it took Landau and Rosenbusch several more years of tinkering before they could nail down the first high-resolution structure of the protein (*Science*, 12 September 1997, p. 1676). Now, however, thanks to decades of painstaking work by a small band of researchers, the technique is beginning to hit its stride.

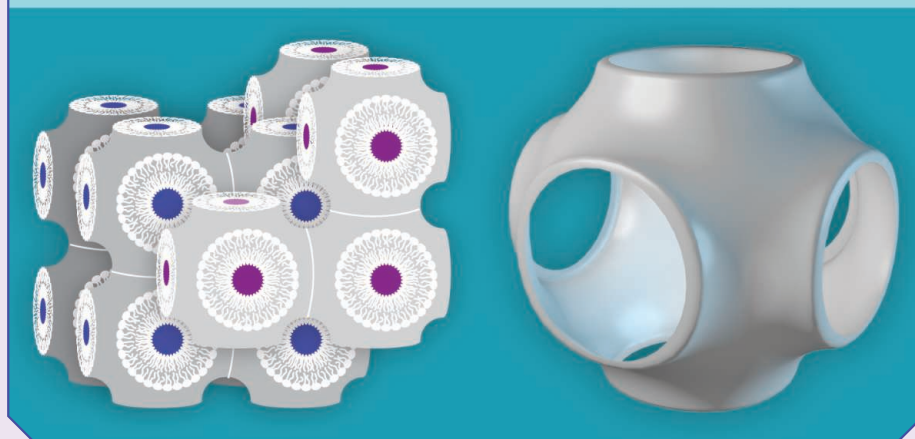
Fits and starts

LCP wasn't the first technique that crystal growers used to enforce order among membrane proteins. Nor is it the most common approach even today. Both of those honors go to a technique that uses soaplike detergents to purify membrane proteins and get them out of cell membranes, a necessary step for getting them to crystallize.

Detergents contain two different kinds of compounds joined at the hip. On one end are hydrophilic groups, which readily associate with water. On the other end are fatty hydrocarbon chains. Dump the detergents into water in the right conditions and they form micelles, tiny spheres with the hydrophilic portion facing out into the water and the fatty hydrocarbon tails pointing inward to minimize their interaction with water. When lipid molecules, which have different hydrophilic groups linked to hydrocarbon tails, are added, the mix can form “bicelles” shaped like tiny disks made from a combination of the lipids and detergents, all with their hydrophilic portions facing out into the water.

Membrane proteins also typically contain one portion that prefers to associate with fatty membrane molecules, and two others that gravitate to the watery environment outside or inside the cell. So if you toss a membrane protein into a solution with micelles or bicelles, the water-fleeing portions of the protein will wedge themselves into the friendly confines of the hydrocarbons, stabilizing their structure. Add millions of copies of the same membrane protein, and if you're lucky they will all orient themselves the exact same way, making it possible for them to pack into an orderly crystal.

Protein-Friendly Geometry



Shapely. In a lipid cubic phase structure, lipid molecules form a hollow framework (right) that extends to form a 3D grid around water channels (left, purple and blue).

That strategy works in some cases. But often it goes spectacularly wrong. Sometimes the detergents are too harsh and rip apart the proteins. The tightly curved spherical micelles can wrench the proteins out of their normal shape, and subtle temperature differences can wreak havoc with bicelles.

Back in 1992, Landau thought LCPs might be a gentler option. LCPs have a gradually curving framework that arranges itself into a 3D grid surrounding a network of watery channels (see figure, above). Landau and Rosenbusch hoped the LCPs' combination of the lipid framework and watery channels would keep both parts of membrane proteins happy and the 3D grid arrangement might help orient them all in the same direction.

But LCPs “can be a hassle to work with,” says Martin Caffrey, an LCP expert and membrane protein crystallographer at Trinity College Dublin. LCP is a clear goop with the consistency of toothpaste, Caffrey explains. While crystals can simply be filtered out of liquid detergent solutions, finding nearly invisible flecks of protein crystals inside the LCP is a real pain. The bR crystals were an exception: Their bright pinkish purple color made them stand out. “I was extremely excited,” Landau says of the day in 1995 when he first spotted the tiny neon

crystallites. “It was obvious to me that our concept had worked.”

Of course, Landau and his colleagues still didn't have a structure. And their next problem was the x-ray beams produced by synchrotrons. These stadium-sized machines fire a staccato burst of densely packed x-rays at their targets. By tracking the way the x-rays diffract off their target, researchers can deduce the atomic structure of the material.

The trouble was that the LCP-grown bR crystals were significantly smaller than those produced in detergent micelles. Most synchrotron beams at the time were 100 microns across, or more—twice the width of the bR crystals. That meant that most of the x-rays in the beamline would whiz right by the bR crystallite and contribute nothing to the diffraction pattern.

Then fortune smiled: The newly built ESRF synchrotron in Grenoble, France, had just opened its first microfocus beamline for work on just such tiny crystals. Landau and Rosenbusch applied for time on the beam, got it, and quickly nailed

down a crisp diffraction pattern for the protein. “This was an extraordinary breakthrough,” Caffrey says.

The question was whether the approach would work for other membrane proteins. Through the late 1990s, Rosenbusch, Landau, and others produced a string of successful x-ray structures with other colored membrane proteins, such as the greenish photosynthetic reaction center. “After that it got quiet,” Caffrey says. Growth conditions that produce protein crystals in LCP invariably trap myriad tiny bubbles in the gel as well, making it even harder to pick out the crystals, if they were there at all.

Caffrey, then at Ohio State University, Columbus, set out to speed things up. In 2000, he and Vadim Cherezov, a postdoc, set about inventing new tools to speed the discovery of crystals in LCP. One was a “sandwich plate” that squished blobs of the clear goop between two glass plates, to make crystals easier to spot under a microscope. Another was a

tute. There he met Raymond Stevens, a renowned structural biologist. After inviting Cherezov to give a seminar on LCP, Stevens asked him to join his group.

“Something that no one has ever seen”

The new landing spot was an ideal fit. At the time, Stevens was collaborating with Brian Kobilka, a biochemist at Stanford University, on attempts to crystallize membrane proteins known as G protein-coupled receptors (GPCRs). GPCRs are one of medicine’s most important sets of membrane proteins, as they transfer chemical signals from outside cells to G proteins inside cells. The G proteins, in turn, launch a variety of molecular dominoes that govern everything from your heart rate to your sense of smell.

By the mid-2000s, Kobilka had managed to grow crystals of a GPCR known as the β_2 adrenergic receptor (β_2 -AR)—a cell-signaling component involved in everything from heart muscle contraction to digestion—

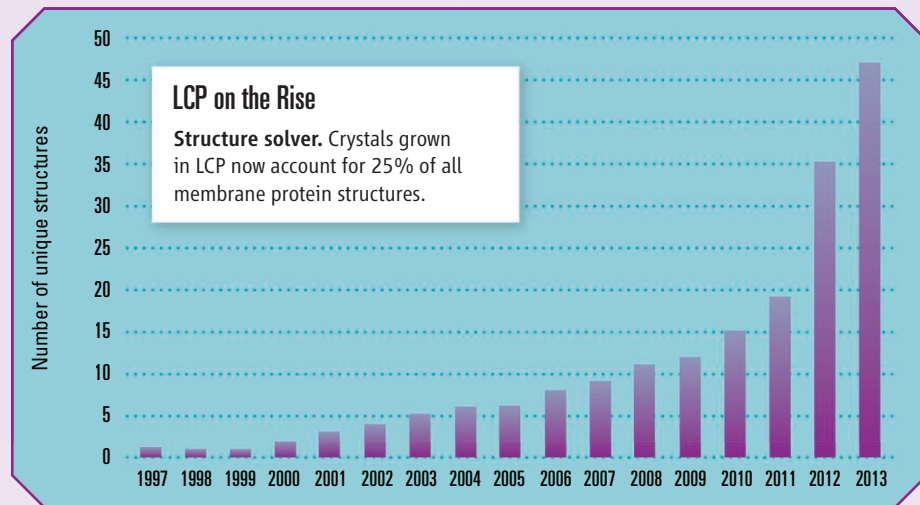
Nature, was one of the first crystal structures of a GPCR. The crystal diffracted to 3.4 angstroms, a resolution that reveals most of the protein’s amino acids.

In hopes of seeing even more detail, Kobilka and colleagues tried another tack. They clipped off a particularly unwieldy portion of β_2 -AR and replaced it with an orderly protein called T4 lysozyme, and grew those hybrids in bicelles. This got them crystals that diffracted to 4.2 angstroms. So they sent a batch of these hybrid membrane proteins to Stevens’s lab. After a few months spent optimizing the LCP conditions, Cherezov produced high-quality crystals, and the researchers took them to the microfocus beamline at the Advanced Photon Source at Argonne National Laboratory in Illinois. The result was a 2.4 angstrom resolution structure (*Science*, 23 November 2007, p. 1258), which *Science* named one of its top 10 breakthroughs of the year.

Next, Kobilka wanted to see if he could get the structure of a GPCR bound to its G protein mate, which would show the GPCR’s conformation in its “on” state. But Stevens, and his postdoc Cherezov, wanted to explore the broader landscape of GPCRs; humans alone have an estimated 800 varieties. So Kobilka teamed up with Cherezov’s former mentor, Caffrey. The G protein turned out to be a behemoth, roughly twice as big as the GPCR. That made it too big to fit into the 50-angstrom-wide watery channels in the LCP. Kobilka hoped to find a way to make the channels bigger.

Back when Caffrey was at Ohio State, he had experimented with dozens of different lipids, charting their effect on the shape and size of the LCP network. He told Kobilka he thought they could widen the channels by replacing the conventional lipid in LCP, known as monoolein, with a shorter chain lipid known as 7.7 MAG.

Caffrey was right. In 2011, using 7.7 MAG for their LCP, along with other changes, Caffrey, Kobilka, and their colleagues were able to get crystals of the complex and work out the structure. “There have been three to four times in my career where I have seen



robot that automated the mixing of different lipids, salts, and buffers needed to crystallize each protein.

Despite a couple of years of rapid progress, LCP efforts nearly ground to a halt again in 2003 when Caffrey was recruited away from Ohio State to form a group dedicated to LCP and membrane protein crystallography at the University of Limerick, in his native Ireland. U.S. science funding agency rules stated that Caffrey was unable to take his robot and other equipment that had been paid for by U.S. taxpayers. Cherezov faced an uncertain future as well. But things took a welcome turn when Cherezov went to San Diego, California, to visit a friend who worked at the Scripps Research Insti-

in conventional lipid micelles. But the crystals were poor and didn’t diffract well, Kobilka says. Like many other membrane proteins, β_2 -AR is a Janus molecule. The part that prefers to nestle within the fatty membrane usually keeps an orderly and stable structure. But the section that protrudes into the watery surroundings flops around like a flag in the wind. Kobilka’s lab was struggling to find ways to stabilize those floppy portions to ensure that all copies of the protein lined up in the same manner inside a crystal. The researchers got partway there by adding copies of an antibody that grabbed part of the floppy portion of the β_2 -AR and held it in place. Then they grew the protein-antibody complexes in bicelles. The result, published in

CREDITS (TOP TO BOTTOM): (ILLUSTRATION) V. ALTOUNIAN/SCIENCE BASED ON PROTEIN STRUCTURE FROM BRIAN KOBIKA; (DATA SOURCE) VADIM CHEREZOV/THE SCRIPPS RESEARCH INSTITUTE

Gatekeepers. Membrane proteins control the chemical traffic into and out of cells and account for more than half of all drug targets.

something that no one has ever seen before. It was very exciting,” Kobilka says. Caffrey agrees. “It was an extraordinary achievement,” he says of Kobilka’s structure of the complex, which helped earn Kobilka a share of the 2012 Nobel Prize in chemistry. “The cubic phase was just part of it, but an important part.”

LCP’s success has been equally important for Stevens. In collaboration with Cherezov, who has since moved into his own faculty position at Scripps, Stevens’s lab has now solved 16 of the 24 GPCR structures completed to date. The collection now represents four of the five major families of GPCRs.

Stevens, Cherezov, Caffrey, and others recently made another leap forward when they adapted a beamline at the free-electron laser (FEL) at the Center for Free-Electron Laser Science in Hamburg, Germany, to solve structures of LCP-derived crystals of membrane proteins with unprecedented efficiency (*Science*, 20 December 2013, p. 1521). FELs represent the latest in synchrotron technology, able to produce x-ray beams that are tighter and pack more than 1 billion times more photons into a given area than ever before. The beams are so powerful, in fact, that they vaporize crystals as soon as they hit them. But because the x-ray photons are traveling at the speed of light, they still manage to diffract well before the slow-moving atoms in the crystal explode outward.

The trick is zapping enough crystals to build up sufficient data to solve a protein’s structure. In 2011, researchers led by Henry Chapman at the Center for Free-Electron Laser Science and Petra Fromme and Uwe Weierstall at Arizona State University, Tempe,

had designed a device for injecting detergent laden with membrane protein crystals into an FEL beamline and showed the setup produced enough diffraction data for the team to solve the structure of an abundant membrane protein. But the technique was a huge waste of crystals. FEL beamlines don’t shine a continuous beam of x-rays. Rather, they send them in dense packets 120 times a second. In between those bursts is essentially dead space that produces no data. To ensure that the x-ray bursts would hit enough crystals, Chapman’s team had to spray in a steady stream of the detergent-and-crystal mixture. The x-ray packets hit only about one crystal in 10,000; the others produced no data. “It’s hugely wasteful” and thus can’t be used with most membrane proteins, which can be harvested only in tiny amounts, Caffrey says.

The LCP aficionados asked Fromme and her injection-builder colleagues to remake their injector to work with the LCP gel. A redesign worked. When the thick LCP goop is pushed through a tiny injector nozzle, it forms a continuous “stream” at a much lower velocity than the previous liquid stream, much as toothpaste emerges from a tube more slowly than a jet of water from a hose. The result was that far more crystals were hit by x-ray packets and the crystal losses were reduced between 100- and 1000-fold.

That triumph should help LCP’s successes continue to roll in. Cherezov notes that in the past 2 years, structural biologists have solved more than 25 unique membrane protein structures with LCP—more than in all previous years combined. LCP-aided structures now account for 25% of all solved membrane structures, a fraction that is growing rapidly.

That doesn’t mean the membrane protein crystallography challenge has been solved. “LCP is not a panacea,” as it still doesn’t work with some of the larger protein complexes, Cherezov cautions. But clearly, Stevens says, the logjam has broken. “For single membrane proteins, for the most part, if we want to get a structure we can get it,” he says. With drug-makers now turning to membrane protein structures to identify novel targets for new classes of drugs against everything from pain and depression to heart disease and migraine headaches, LCP’s success may soon make a difference in millions of peoples’ lives.

—ROBERT F. SERVICE

REVIEW

Cutting-Edge Techniques Used for the Structural Investigation of Single Crystals

Judith A. K. Howard^{1*} and Michael R. Probert²

X-ray crystallography has become the leading technique for studying the structure of matter at the atomic and molecular level. Today it underpins all sciences and is widely applied in industry. It is essential in the development of new materials. The technique is very powerful, and the range of materials that can be studied expands as new technologies evolve and are applied in innovative ways to structure solution. It is now possible to record vast amounts of diffraction data in seconds electronically, whereas it took days and months by photographic methods 30 to 40 years ago. Single crystals can be created in various ways; they can be produced from compounds that are liquids or gases at room temperature, and complete molecular structures can be presented within minutes. This short review presents recent developments that are appropriate to the single-crystal x-ray studies of chemical and materials sciences.

One of the most important scientific tools to emerge from the 20th century is x-ray crystallography. Because the chemical and physical properties of a material depend on its structure, the three-dimensional results derived from a crystallographic study are of enormous importance in the overall characterization of any new material. In recent decades, this technique has also revolutionized the understanding of molecular biology. The centenary celebrations in 2013 for the ground-breaking discoveries of W. H. and W. L. Bragg (1) provide an appropriate point to look at the modern techniques in use today. This short review will concentrate on single-crystal x-ray diffraction methodologies, referencing new instrumentation, sources, and computational tools. We will assume a basic understanding of the single-crystal method and refer the novice reader to some introductory texts on the x-ray experiment for collecting diffraction data (2, 3).

X-ray crystallography experiments have traditionally required single crystals; today, however, there are pioneering studies in the use of multiple crystals (4) with methodologies and programs to interpret data recorded from twinned or multi-crystal samples (5). Samples are no longer required to be crystalline and stable at room temperature, and many single crystals have been grown from liquids by controlled variation of the temperature (6) or the careful application of pressure (7) (Fig. 1). We shall start by describing some methods for crystallization, linked to the appropriate instrumentation, followed by further instrument and

x-ray source developments, and finally explore new computational methods.

Crystallization and Crystal Mounting

Crystals can be grown in the laboratory from solution, by evaporation of the solvent, by cooling, and by balanced-diffusion experiments. The single crystal required for a diffraction study is selected by visual inspection, normally under an optical microscope, from the batch of crystals grown. This crystal is then mounted and supported rigidly during the collection of three-dimensional diffraction data. All crystals are mounted, by various means, onto a goniometer head (Fig. 2), a device with at least three degrees of freedom that allows the crystal to be centered in the x-ray beam. Many methods have been developed for mounting crystals that can be handled in more or less ambient conditions in preparation for the x-ray experiment. Of particular note is the use of perfluorinated oils, which has facilitated fast, reliable mounting of unstable, as well as routine, samples. In this approach, crystals are “fished” from the mother liquor into an oil-filled fiber loop, and thus are suspended in the inert oil. This method is much easier than the previous one of sealing crystals inside thin-walled glass capillary (Lindemann) tubes. The older method, using Lindemann tubes, is still employed if the sample exhibits rapid decomposition through solvent loss. This method allows a local positive pressure of the crystallization solvent to be created, by sealing the tube with the crystal and a drop of the solvent. Crystal mounting techniques are still being developed using ever-more exotic materials, such as graphene, to reduce background during the diffraction experiment.

Crystallization from the melt of a material, by zone refining—a localized heating method—has

been employed successfully in producing large single crystals for use as semiconductors—for example, in the electronics industry (8). On a laboratory scale, zone refining is useful when dealing with materials that have low melting points and exist as liquids at room temperature (9). The laboratory methods used for the growth of crystals from liquids are outlined below. If the crystals are to be grown from liquids by cooling or by pressure, then the container in which the crystal growth takes place is also the mount used for the crystallographic characterization. When crystal growth is to be controlled by temperature, the pure liquid is sealed in a short capillary tube that is mounted in a metal holder and attached to the goniometer head (Fig. 2, inset). This assembly is then placed onto the diffractometer (Fig. 2), the instrument for conducting the diffraction experiment, and the growing procedure can begin. The contents of the capillary are cooled well below its melting point to give a microcrystalline solid or a glass; the temperature is then raised to just below the melting point, and the process of zone refining begins. This requires carefully controlled temperature changes to produce successive melting and crystal growth. The smallest crystals are melted first, and the larger crystals that persist can then act as seeds for the next round of crystal growth. This process is iterated until a suitable single crystal has been achieved. The task requires considerable skill and patience, but the results are most rewarding. The introduction of the optical heating and crystallization device (OHCD) (10), a targeted laser that allows highly localized heating while the rest of the sample remains below the melting point, improved success rates in this field. It successfully removed much of the chance present in the more primitive methods, which allowed only those with “gifted hands” to produce diffraction-quality crystals from liquids. Many laboratories now use these methods, producing novel and impressive results. Producing crystals *in situ*, which may take several hours of optimization, requires careful control of conditions to give a suitable crystal for diffraction.

Crystal growth from liquids can also be initiated by the application of pressure; this is usually achieved inside a diamond anvil cell (DAC) (11) (Fig. 3). The approach used is similar to the one described above, whereby the conditions are carefully controlled around the sample's melting point. In this case, however, the liquid is pressurized into a solid state, either microcrystalline or a glass, and then the pressure is controlled around the melting point to initiate crystal growth. If a suitable crystal can be grown successfully, the diffraction experiment must then be carried out with the crystal inside the DAC under the conditions in which it was created, often with pressures exceeding several thousand atmospheres. This method can result in the creation of a different polymorph from that obtained on cooling (12). Changes in the protocol for pressurization on the

¹Chemistry Department Durham University, Durham DH1 3LE, UK. ²School of Chemistry, Newcastle University, Newcastle NE1 7RU, UK.

*Corresponding author. E-mail: j.a.k.howard@durham.ac.uk

same sample can also induce different polymorphic forms, which cannot be achieved through other methods. There is an unexplored world of crystalline forms waiting to be discovered through careful control of the sample environment using modern techniques. DACs can produce extremely high pressures up to geological scales to simulate pressures in the mantle and in Earth's interior (13), but these pressures are rarely approached in chemical studies, because excessive pressure destroys the single crystals grown at more moderate pressures.

Crystal growth under nonambient conditions of pressure or temperature has opened up areas of chemistry in which the only methods for structure characterization previously were the various supporting spectroscopies. In addition, it has led to the discovery of novel polymorphs, some of which had been suggested by structure prediction software (14), a field that has recently blossomed with the advent of powerful modern computers. Recent work, attempting to grow cocrystals from combinations of two liquids, has also revealed a further route to undiscovered polymorphs, in cases where only one of the individual components crystallizes rather than the expected cocrystal (15).

X-ray Sources and Detectors

Recent advances in both detector technology and x-ray sources have greatly contributed to the ongoing revolution in crystallography. Sources and detectors are intimately combined with the methods and instrumentation and, therefore, are synergistic with developments in the field. Enormously brighter x-ray sources; better, faster detection; and advanced crystallographic software all enable and encourage innovative experiments and novel instrument development. The global crystallographic community and commercial companies work closely to ensure that innovative ideas become affordable instruments or devices.

X-ray Sources

Sealed tube x-ray sources were the commercially available laboratory standard until the early 2000s with diffractometers being equipped with either a copper or a molybdenum metal target (anode). These metals produce x-radiation of mean wavelengths 1.5406 and 0.7107 Å, respectively, and are operated at a power of 1.6 to 3 KW. Other metal targets (Sc, Ti, Cr, Mn, Fe, Co, Cu, Mo, Rh, Ag, Gd, W, Au) have become available, but are much less common and are used for more specialized experiments ranging from single-wavelength anomalous

diffraction (SAD) phasing to high-resolution, high-pressure studies. Rotating anode generators were developed (16) to increase the incident flux at the sample, enabling much smaller crystals to be analyzed by x-ray diffraction. The increase in power was made possible by spreading the heat load on the anode. In these generators, the anode rotates in a vacuum and is internally cooled with water. Rotating anode generators, providing high-brilliance laboratory sources, are now in demand in all areas of crystallography, allowing the study of exotic ma-

terials where crystal size is limited (17). Microfocus optics, using multilayer components to focus the x-rays, were introduced into laboratory sources more than 10 years ago, but only more recently have they been available in reliable and affordable formats (18) and been used routinely by instrument manufacturers for the three most common targets, Cu, Mo, and Ag. The optics are incredibly efficient and, coupled to the latest generation of x-ray tubes, allow high flux densities to be achieved at a fraction of the power of traditional sources (<50 W compared to >6 kW), having a major environmental impact on laboratory energy usage.

In the latest high-intensity laboratory sources, designed for the study of exceedingly small crystal samples, the conventional solid metal anode has been replaced by a liquid metal jet, thus removing the cooling previously required for the anode to be maintained at temperatures well below its melting point. The liquid metal alloy can support a higher electron beam power density than a solid anode, and can therefore generate a much higher x-ray flux. The latest commercially available system uses a liquid gallium-rich (~90%) alloy, coupled with a LaB₆ cathode, producing an x-radiation of wavelength ~1.34 Å. Future developments of this technology are expected to expand the number of available metal alloys for different wavelength applications (19).

The advent of synchrotron radiation for dedicated use as a high brilliance x-ray source in the 1960s is now part of our history, but previously there had been some parasitic use of facilities that were designed primarily for the high-energy physics community (20). There are now more than 40 large synchrotron facilities across the world, and crystallographers are major users of these facilities. Data collection strategies for chemical and materials crystals are generally less complicated than for protein samples, but the intense beams can cause substantial radiation damage to the crystals, and new protocols have been devised to reduce this.

The most recent advance in the area of x-ray sources is the development of x-ray free-electron laser (XFEL) facilities, which provide short, intense, coherent femtosecond x-ray laser pulses with intensities that are many times higher than in current-generation synchrotron sources. The short wavelength (<1 Å) and pulse lengths provided, from 200 down to 30 fs, will be appropriate to study fast chemical reactions, which are too rapid to be captured by other methods. The Linac Coherent Light Source (LCLS) at SLAC Stanford began operation in 2009, SACLA at Spring 8 became operational in 2012,

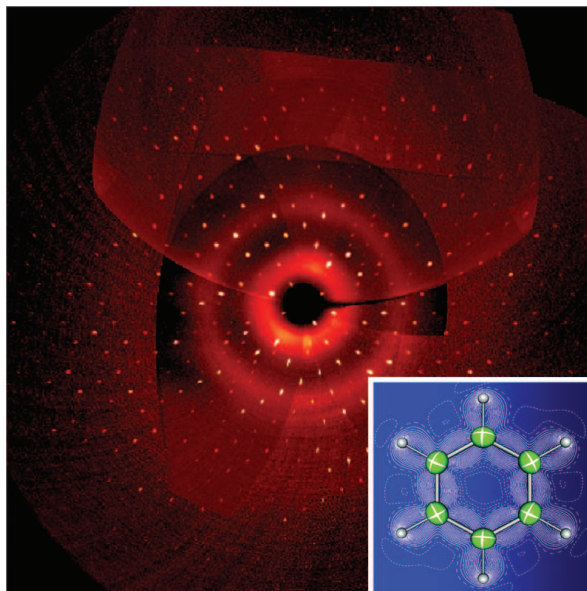


Fig. 1. Precession photograph image and molecular structure of benzene (28).

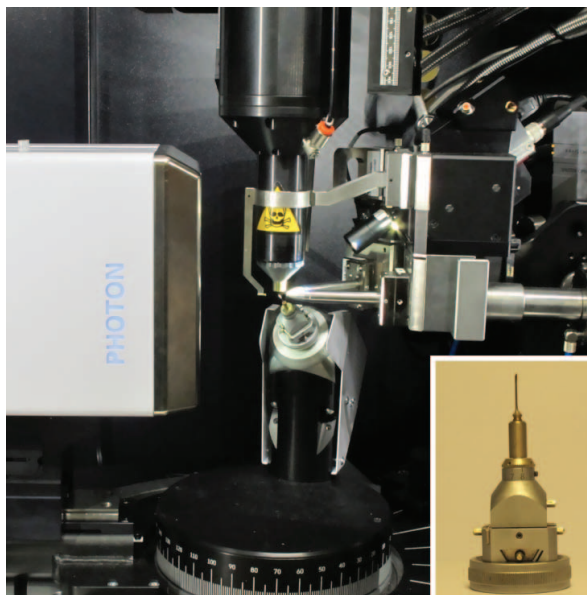


Fig. 2. A modern dual-source, three-circle diffractometer, with large CCD area detector. Goniometer head (inset) allows the crystal to be positioned accurately at the center of the instrument for data collection.

and the European XFEL at DESY (Hamburg) will be online in 2015 (21). Very recent experiments on photosystem II at the LCLS (22) show what can be achieved with XFEL. The emergence of such sources offers very exciting challenges for the future, requiring extremely fast processing and management of vast amounts of data that each experiment will produce on every structure to be studied at these sources in decreasing quanta of experimental beam time. It may, for example, be possible to trigger chemical or biological reactions inside the crystals during their diffraction experiment. Another challenge lies in merging and interpreting data from many crystals that may not all behave in exactly the same way.

Detectors

As x-ray sources became brighter, detector technology necessarily moved in parallel. Huge changes in experimental procedure followed the introduction of charge-coupled devices (CCDs) into laboratory diffractometers in the 1990s. CCDs, the technology in many digital cameras, allow diffraction images from large phosphor sensors to be rapidly recorded, decreasing the time required for a standard diffraction experiment from days to hours. The previous state-of-the-art required each reflection in a diffraction pattern to be measured individually with a single point detector. Area detectors had been in use for some time in neutron diffraction studies (23), but only a limited number of protein laboratories were using them for x-ray studies, before the increased demand by the new synchrotron beamlines and subsequent investment in laboratory infrastructure in chemistry and materials science departments. Since then, new developments have brought faster, more sensitive, more reliable detectors with no moving parts into the laboratories and onto facilities' beamlines. The latest developments have introduced solid-state detectors, which allow direct photon counting, continuous readout, and time gating of the detector itself (24). Each subsequent development has enabled experiments that were not feasible with the previous generation, up to the present time where entire diffraction data sets can be collected in a matter of seconds.

The demands of XFEL will drive the next generation of fast x-ray detectors and the software to process the vast amounts of data recorded. The detectors and software will bring us closer to the realization of real-time studies of chemical and biological processes, where subsequent experiments can build up direct observations of these events.

Crystallography at Low Temperatures and High Pressures

Routine, low-temperature laboratory data collections were unusual until the 1980s, by which time only 4% of data sets in the Cambridge Structural Database (CSD) (25) were shown to have data recorded below room temperature. This has risen to 44% of all structures deposited since 1980;

taking deposits since 2000, this number rises to 57%. The introduction of cooling the sample by a stream of cold gas (26) expanded the range of samples that could be studied routinely, allowing air-sensitive samples to be mounted in inert oils, which freeze on cooling. It also enabled the collection of crystallographic data from radiation-sensitive crystals (27). The reduction in thermal motion and diffuse scattering, the increase in the amount and quality of the data, and the stabilization of sensitive samples are all recognized advantages of low-temperature studies (28). The decrease in thermal motion not only improves standard diffraction data quality, but also enables the collection of high-resolution data. These data enable structural studies to move beyond the determination of atomic positions and allow the investigation of a material's full electron density (29–31), resulting in a greater understanding of its electronic characteristics and physical properties. In addition, it is possible to study subtle structural changes by recording data sets over a wide temperature range. This is of particular interest when structural phase changes can be correlated to the sample's macroscopic properties. Understanding the link between the molecular structure and the property of a material is a fundamentally important aspect of today's multidisciplinary studies.

A number of cooling devices are available for use with single-crystal diffractometers, depending on the temperature range required. The most commonly employed device uses a cold inert gas stream, usually nitrogen, which is directed onto the crystal throughout the experiment to maintain a set temperature. This temperature can be varied at controlled rates across the range of the device—for example, from ~80 to ~500 K for modern open-flow nitrogen systems. These devices are highly

successful and the most common in crystallography laboratories across the world. There are also open-flow systems that use helium gas as the crystal coolant (32), providing a temperature range from 15 to 300 K. These devices are less widely used owing to cost of operation, but have the great advantage of allowing access to the sub-liquid nitrogen temperature range (below 77 K). Cooling to ultralow temperatures requires a different approach, as open-flow technologies cannot access this region. A desire to understand fundamental solid-state physics phenomena that occur only at these very low temperatures gave rise to the development and use of closed-cycle cryorefrigerators (CCRs), where the sample is cooled by conduction and thermally isolated under vacuum. The use of these systems has not been large outside of central facilities, but these are enormously powerful instruments, providing unique access to this temperature regime (33). CCRs impose some experimental constraints due to their size, but allow specialized experiments to record accurate, high-resolution, x-ray data over a very wide temperature range—for example, 2 to 300 K when using a three-stage closed-cycle He gas cryorefrigerator. The instruments are not available commercially; they are expensive and time-consuming to build, but uniquely powerful for determining the three-dimensional structure of materials at very low temperatures and for studying phase changes that relate to important macroscopic properties, such as molecular superconductivity and magnetism (34).

DACs, mentioned previously, are most commonly used for high-pressure studies of materials that are crystalline under ambient conditions. They enable the elucidation of structure evolution under pressure variation, analogous to the investigation of structural modifications that accompany changes

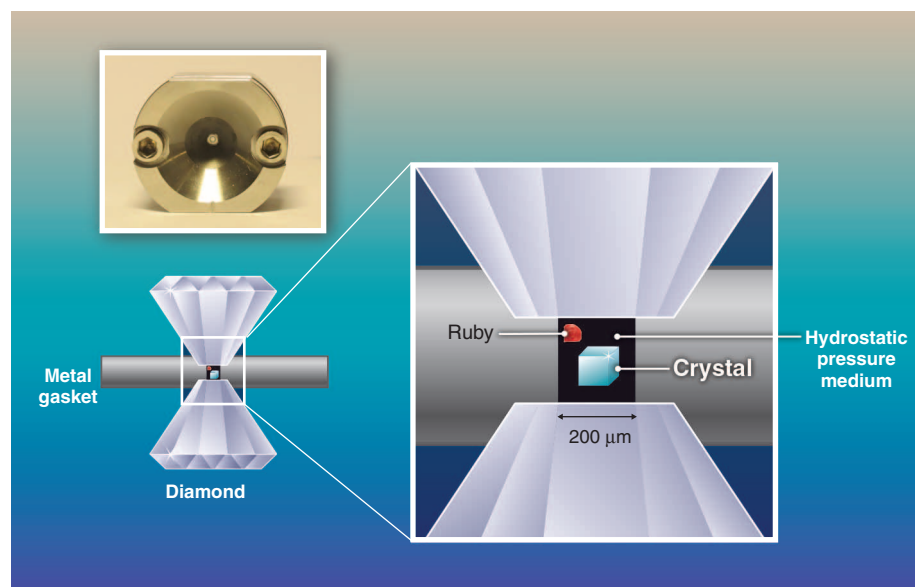


Fig. 3. A diamond anvil cell (DAC) (top left) capable of pressures >100 kbar, with operational schematic (below).

in physical, optical, electrical, or magnetic properties that are followed today over wide temperature ranges (28). Unfortunately, the data that can be recorded in a high-pressure experiment using DACs are restricted, because the cell body obstructs the diffracted x-ray beams. Modifications to the original DAC design alleviate some of these problems (35), but the diamonds and the bulky superstructure of the cells themselves create an obvious physical limitation. In most high-pressure experiments, a tiny ruby chip is enclosed with the crystal and its hydrostatic medium inside the small gasket of the cell (Fig. 3). This is used to determine the pressure within the cell, as the changes in the fluorescence spectrum of ruby with pressure have been extremely well calibrated. Data reduction requires careful attention because there are strong reflections from the diamonds and scattering from the cell body materials. This has driven the development of programs to apply “masking” to the data set and to correct the data adversely affected by the DAC scattering or diamond reflections (36). A portable, moderate quartz pressure cell (QPC) has been designed that uses a moderately thick-walled quartz capillary tube as the pressure chamber to contain an optically visible crystal and to enable single-crystal data collection at pressures of up to 1 kbar created by the application of gas or liquid (37). The use of a gas to apply the pressure also enables the investigation under non-ambient atmospheres (38–40). The QPC system has operational and data-reduction advantages over the DACs, but in a limited pressure range, albeit one that fills the gap between ambient pressure and the lower range of DACs for single-crystal samples.

These experiments have led to the full characterization of materials that exhibit abnormal behavior upon the application of pressure, such as

negative compressibility, and also enable the monitoring of pharmaceutical active ingredients under the moderate pressure conditions used during tablet formulation.

Photocrystallography

There is considerable scientific and industrial interest in this area of structural chemistry, which aims to determine the full three-dimensional structure of photoinduced species in order to understand the molecular and macroscopic properties with respect to the ground state and excited states of the material. Mapping often subtle structural changes induced by light, heat, pressure, magnetism, and electric current with respect to time is fundamental to our understanding of reaction mechanisms, but achieving this in the solid state is a considerable challenge. Pioneering work (41–44) has established the techniques required for these experiments, and we are approaching true “time-resolved” studies with the latest x-ray (XFEL) sources (45, 46). Recent decades have seen an explosion in optical and optoelectronic devices that exploit switchable materials, and it is necessary to understand these molecular and electronic processes in detail to design and create new materials that are stable and robust to thermal/photocycling (47). The conversion ratios between states (photoexcited to ground) is commonly rather small in many solid-state reactions, and ways to enhance this for usable materials is one goal of this growing research area.

Photoactivation can be reversible or irreversible, short- or long-lived, and each type of “switch” presents challenges for the crystallographer to achieve high-resolution structures and requires different experimental methodologies. The very fast (femtosecond) chemical reactions require the latest, brightest x-ray sources and very fast lasers,

whereas some long-lived reactions can be followed at synchrotron sources when observing in the micro-to-millisecond time frames (48). Although much research has been published for decades on photochemical reaction studies by optical spectroscopies, the molecular detail and high atomic resolution of crystallography have been missing. We can now perform the complementary diffraction experiments to enhance our understanding of these fundamental, but highly important, chemical and biochemical processes.

Photoswitchable materials include spin crossover (SCO) compounds (49–52), photo-(thermo)-chromic materials (53–56), photocycloaddition (PCA) compounds (57, 58), and photoisomeric compounds (59, 60), and these have found application variously in optical storage materials, light and pressure molecular switches, sensors, molecular wires, logic gates, and imaging (Fig. 4). Photocrystallographic experiments strive to achieve high-resolution diffraction data from the ground state and subsequent excited states in the same single crystal, which requires a conversion of at least 10% and no serious degradation of the crystal in the process of excitation. There are many examples of these successful experiments in the literature, but achieving the reverse process in a single crystal can be challenging or impossible, depending on the chemical reaction.

Crystallographic Software

Structure solution and refinement algorithms have advanced with the increasingly accurate, higher resolution, x-ray data now recorded, largely free of systematic errors. Several structure solution and refinement packages (61–64) are now available, all being actively developed to interpret more complete structural data and reduce possible errors in the final model. Structural descriptors that go beyond the spherical atom model (65), and allow the full electron density elucidation of compounds, have become more mainstream, and further developments in this field now require only moderate-resolution data. The advances in both detector and source technologies outlined above have driven the development of data collection and processing software, with many central facilities using robotic mounting and centering routines (66). Data from these are often relayed to automatic processing software that will attempt to produce a near-finished molecular model. Combining these functions moves the more routine structural interrogations into the realm of full automation. This allows the crystallographic experts to concentrate on the more challenging systems, such as multivariable experiments, obscure sample environments, low-resolution data, incommensurate crystals, and quasicrystals. One further area seeing rapid development is in the strategies to record, process, and interpret data from experiments designed to follow reactions in real time (46).

A recent revolution in structure solution that is important to mention is the introduction of the charge flipping algorithm (CFA) (67). This is a dual-space

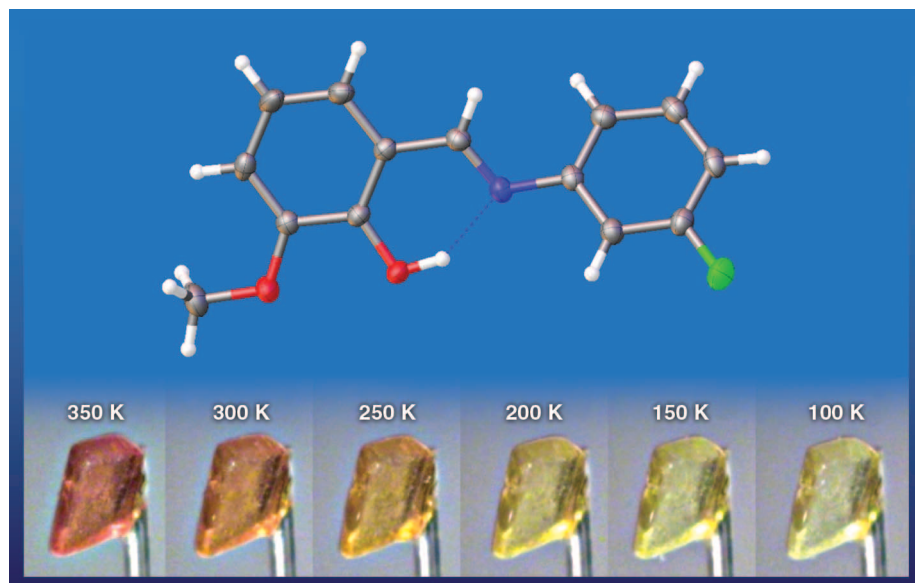


Fig. 4. Molecular compound that undergoes a minor conformational change with temperature. (Below) The crystal of the compound, showing obvious thermo-chromic behavior between 100 and 350 K.

phasing algorithm, utilizing the fundamental knowledge that electron density in a crystal structure must be positive. The method has rapidly become a popular alternative for data sets where traditional methods fail (68). CFA has a major advantage over traditional solution methods, as the space group of the structure does not need to be determined before use. It is the only structure solution method that is currently extensible to systems where the full symmetry of the system is described by $3 + n$ dimensions.

The Future

Chemical and materials sciences lie at the basis of the next generation of smart materials, fabrics, and devices, and x-ray crystallography is fundamental to their design and successful application. The use of crystallography in online analysis will continue to be an essential industry tool, and instruments will become faster, smaller, more portable, and applicable in the field for important health problems in remote areas and the developing world. Concurrently, the development of new powerful x-ray sources for the laboratory, as well as at global central facilities, will enable new discoveries at higher resolution by using much smaller crystals, and importantly, these experiments will use much less of the crystalline materials in the studies, whether pharmaceutical compounds, precious metals, or the rare chemicals that are needed in modern electronics. Recent discoveries at the molecular level for smart materials with clever magnetic and electrical properties (e.g., single-molecule magnets) require extensive dynamic structural studies to explain the subtle molecular changes under applied external fields so that these changing properties can be exploited in the next generation of devices. Taking crystallography to other planets, most recently Mars, has challenged the imagination of crystallographers, engineers, mathematicians, and many other materials scientists, with staggering results, and we can expect to see more missions that take remote-controlled laboratories to distant places—missions that were unimaginable a few years ago. The collaboration of scientists developing portable x-ray sources, fast, sensitive detectors, intelligent robots, innovative software, and data analysis methods will find many applications and challenges for crystallographers in the decades ahead. Fortunately, crystallography has a long history of sharing ideas, experiences, expertise, methods, and software for the common good (69, 70).

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REVIEW

Developments in X-ray Crystallographic Structure Determination of Biological Macromolecules

Elspeth F. Garman

The three-dimensional structures of large biomolecules important in the function and mechanistic pathways of all living systems and viruses can be determined by x-ray diffraction from crystals of these molecules and their complexes. This area of crystallography is continually expanding and evolving, and the introduction of new methods that use the latest technology is allowing the elucidation of ever larger and more complex biological systems, which are now becoming tractable to structure solution. This review looks back at what has been achieved and forward at how current and future developments may allow technical challenges to be overcome.

Macromolecular crystallography enables the three-dimensional (3D) structures of large biologically interesting mole-

cules to be determined. Structures of proteins and nucleic acids determined by macromolecular crystallography are vital for elucidating protein function



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Macromolecular crystallography enables the three-dimensional (3D) structures of large biologically interesting mole-

cules to be determined. Structures of proteins and nucleic acids determined by macromolecular crystallography are vital for elucidating protein function

and intermolecular interactions and for improving our understanding of basic biological and biochemical mechanisms and disease pathways. Their immediate practical application is in the design of pharmaceuticals, in which they play a central role in drug discovery.

This branch of crystallography has dramatically advanced over the past 80 years since the 1934 initial observation of diffraction from crystals of a small protein, pepsin, and the first protein struc-

ture determination (myoglobin) (Fig. 1A) in 1958. Haemoglobin followed, and then in 1965 the first enzyme structure, lysozyme (Fig. 1B), was solved. The recent characterization of the entire ribosome (Fig. 1C) revealed one of the essential machines of life, comprising a vast complex of molecules consisting of ~280,000 nonhydrogen atoms; more than 2.5 orders of magnitude larger than the 1260 in myoglobin. The field has been awarded 28 Nobel Prizes—starting with father-and-son team William Henry and (William) Lawrence Bragg in 1915—with the latest being the 2012 Chemistry Prize won by Kobilka and Lefkowitz for studies on G protein-coupled receptors (GPCRs), crucial cellular sensors

for signaling proteins and hormones. These Nobel Prizes signal the effect that crystallography has had and continues to have in the world of cutting-edge research.

Macromolecular crystallography was born with the pivotal discovery by Bernal and Crowfoot (1) that pepsin crystals retained their order if kept hydrated in a capillary tube sealed at each end during x-ray diffraction experiments. Unlike the crystals formed by inorganic or small organic compounds, macromolecular crystals can contain up to 90% solvent surrounding the molecules. The intermolecular interactions supporting the crystalline lattice are weak. The success of diffraction experiments

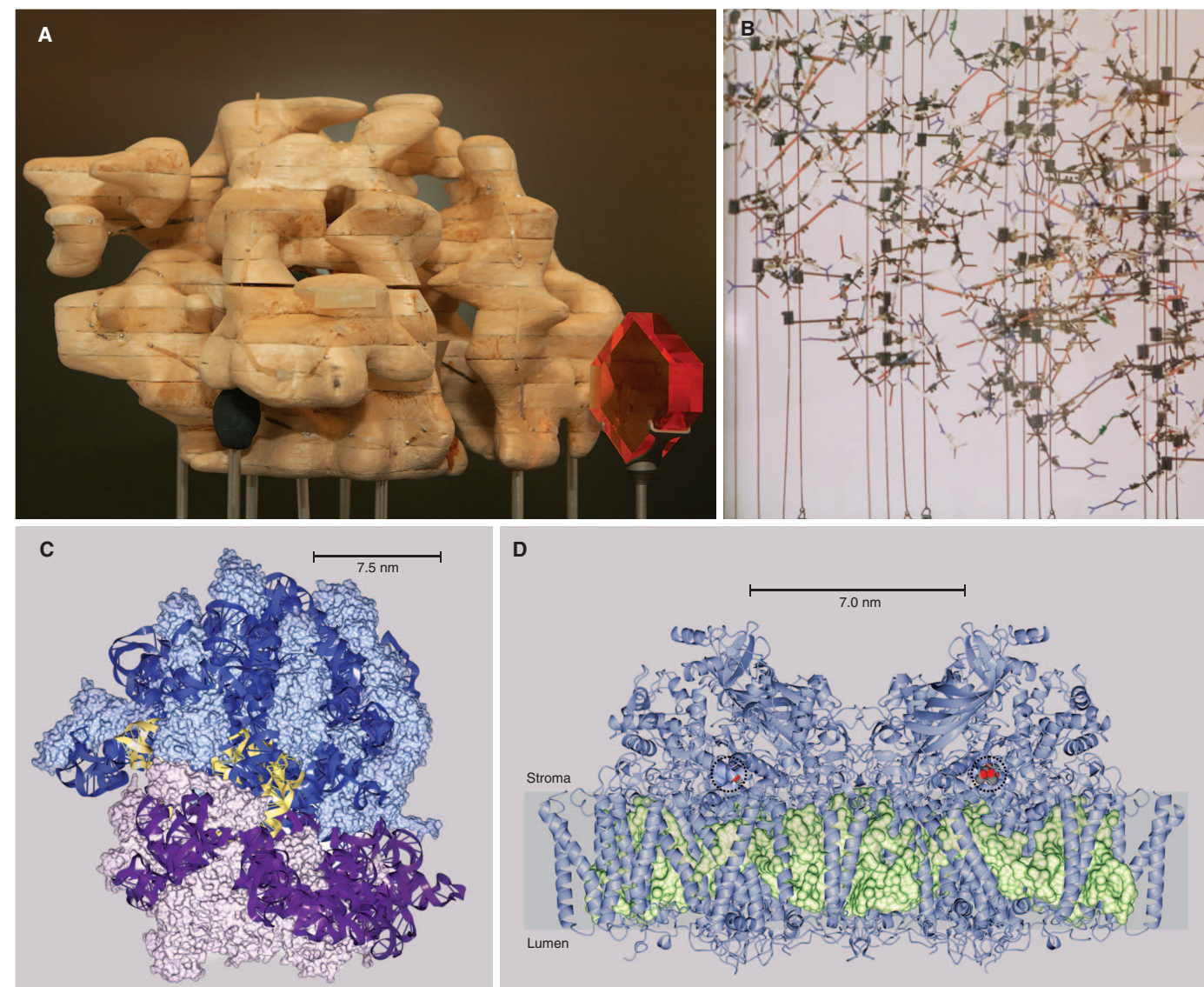


Fig. 1. Visualization of macromolecular structures. (A) Balsa wood model of myoglobin at 5 Å resolution (45) and a model of a monoclinic crystal, made by H. Scouloudi, 1969. (B) Wire model of lysozyme structure (39). Model constructed by W. Browne and M. Pickford circa 1965. Refurbished by A. Todd and Unicol Engineering of Headington, Oxford, UK. Blue, nitrogen; red, oxygen; black, carbon; yellow, sulfur; and gray, hydrogen bonds. (C) Ribosome 70S particle at 3.5 Å resolution (46). 30S subunit and tRNA, PDB entry 2wdk;

50S subunit, PDB entry 2wdl. The 30S subunit is shown in purple (pale for protein, dark for RNA) and the 50S subunit in blue (pale for protein, dark for RNA). The tRNA is in gold. Figure made with CCP4mg (47). (D) Photosystem II at 1.9 Å resolution. PDB entry 3arc (48). The protein is shown in blue and the chlorophylls in green. The oxygen-evolving cluster is depicted as spheres and highlighted by dotted circles, and the membrane bilayer is indicated by a shaded box. Figure made with CCP4mg.

critically depends on crystalline order, which usually deteriorates if the crystals are allowed to dehydrate. Many of the technical challenges in the field arise from this property of protein crystals.

Crystallographic macromolecular structures are time and space averages over the many millions of macromolecules within the crystal. A “large” protein crystal is typically smaller than 100 μm in all three dimensions. For an average-sized 5-nm-diameter globular protein, such crystals would contain $\sim 10^{15}$ molecules. The dynamical behavior of the molecules within a crystal allows only a limited sampling of the conformational space of the protein because the crystallization conditions bias the behavior. Better information on dynamical properties is required to fully understand protein-protein interactions and pathways. Techniques to address this issue are being explored with the aid of newly available technology, and current approaches are described elsewhere in this issue (2).

For the past 20 years, over 95% of macromolecular structures have been determined from crystals held at cryotemperatures ($\sim 100\text{ K}$) because the rate of radiation-induced damage is lower by a factor of ~ 70 compared with room temperature (3). Although 100 K is far from physiologically relevant temperatures, it is clear from structural studies of the same proteins at different temperatures that the overall fold of the alpha-carbon amino acid chain is temperature independent. More ordered water molecules can be located in structures determined at cryotemperatures, and alternative conformations of side chains tend to be better defined. This is because the dynamic disorder in the protein is “frozen out” and the observed substate populations reveal only the static disorder. Because these detailed observations are not necessarily physiologically relevant, ideally structures would also be determined at room temperature if this could be conveniently expedited.

Currently, some promising new developments in macromolecular crystallography are unfolding. Future growth areas summarized below are membrane protein crystallography, and room-temperature data collection both at synchrotrons and at the recently introduced x-ray free-electron lasers (XFELs).

The Pipeline

The deployment of new technology and methodology is continually streamlining the pipeline involved in macromolecular structure solution (Fig. 2) and improving the success rates for challenging cases. However, the major bottleneck remains the growth of diffraction-quality crystals.

Before crystallization can be attempted, sufficient quantities of protein must be purified, usually as recombinant material from bacterial, yeast, insect, or mammalian cells. Expression systems have become high throughput as a result of more rapid and reliable cloning tools and the more widespread use of automation and bioinformatics. These developments permit better-informed and extensive screening of expression vectors, protein sequences, and hetero-

logous host cells (4). It can still be a labor-intensive and time-consuming task to optimize the system to produce enough protein for crystallization trials. However, with recent methodological progress, the structures of an increasing number of proteins that were historically viewed as challenging (e.g., membrane proteins, posttranslationally modified proteins, and protein complexes) are now being solved.

An important development has been the use of autotrophic strains for the incorporation of seleno-methionine into recombinant protein, because the selenium allows the structure to be experimentally phased by the multiwavelength anomalous dispersion (MAD) method (5).

To maximize the chances that crystals will grow, the protein must be as homogeneous and pure as possible, so it must usually be in a single oligomeric state. Large losses of protein may be experienced during purification, but this step is vital for successful crystallization. Techniques for assessing protein purity have advanced considerably, and a variety of methods are now used, including dynamic light scattering and coupling of size-exclusion chromatography with multiangle laser light scattering. These reveal whether a protein sample is monodispersed and homogeneous, often giving a good indication as to whether it might crystallize.

Although the parameters governing the process of protein crystallization are now better understood through research into crystallogensis, it is not yet possible to predict the conditions under which a particular protein will crystallize. Thus, the approach is still to coarse-screen a wide range of chemical conditions—such as buffer type, temperature, pH, protein concentration (typically 10 to 20 mg/ml), cocktails of detergents if it is a membrane protein, precipitants (organic solvents, salts, and polymers), presence or absence of divalent cations, and additives—in the hope of obtaining a few hits. Screening on a finer grid that samples around these promising conditions then allows optimization, which may result in diffraction-quality crystals.

Crystallization robots that can routinely dispense low-volume drops (as low as 50 nl protein + 50 nl of precipitant solution) permit thousands of conditions to be coarse-screened. This has greatly increased the likelihood of crystallization conditions being found given limited protein volumes; for instance, with 150 μl of protein, ~ 1500 trial drops of 100 nl + 100 nl could be tested in slender 96-well plates holding two conditions per well. Larger volume than the minimum 50 nl is usually dispensed, because scaling up crystallization conditions from such small drops can be problematic due to changes in surface-to-volume ratios. The trays are typically kept at a constant temperature (e.g., 4°C or 20°C) in “crystal hotels” equipped with imaging devices that automatically photograph the crystallization drops at regular intervals, and these images can then be scored using automated crystal recognition software. Thus, much of the drudgery has been removed from the search for suitable conditions. The successful development

of such automated systems owes much to the investment of resources and time made in structural genomics centers in the early part of this century.

Once a crystal has been obtained, it must usually be manually harvested from its growth drop before

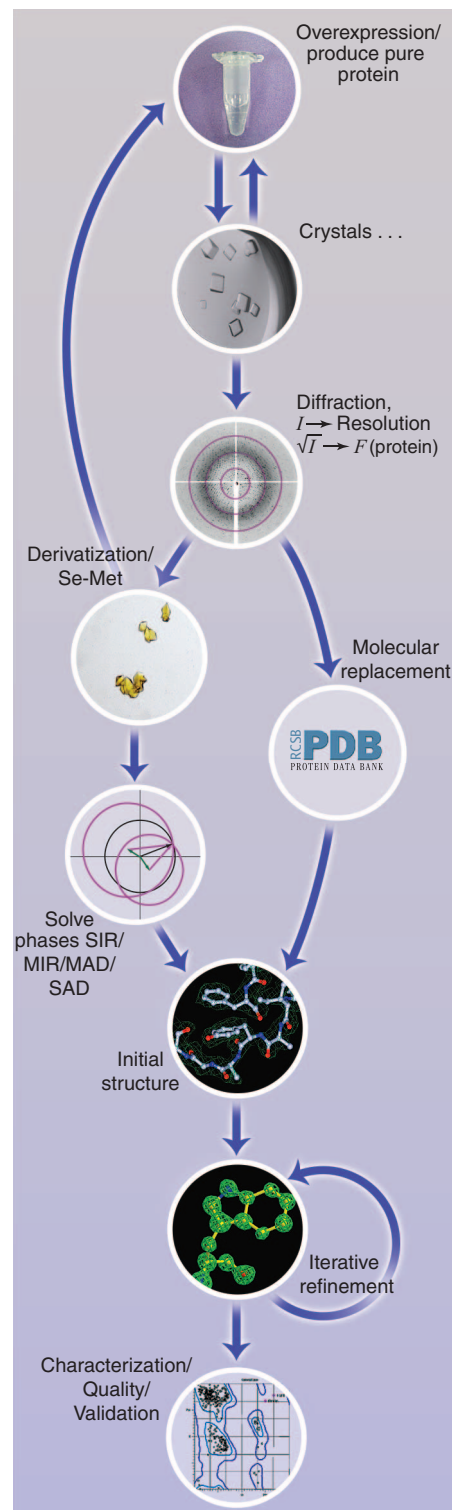


Fig. 2. Diagram showing, from top to bottom, the pipeline for macromolecular structure solution.

being irradiated with x-rays. Successful vitrification (Fig. 3) of the crystal for data collection at cryotemperatures generally requires the presence of cryoprotectants. The flash-cooling of crystals (6), held in cryoloops by surface tension, is a step in the macromolecular crystallography pipeline that has so far proved difficult to automate. Commercial cryoloops are available in a range of sizes and made from rayon, microfabricated polyimide film, and etched mylar, some having integral meshes to support fragile crystals or many small crystals simultaneously. Technically, there is a pressing need for automatic crystal harvesting and sample handling methods to overcome this pipeline bottleneck.

The evolution of storage ring sources to the currently available third-generation synchrotron sources (7) (Fig. 4) in conjunction with fast and accurate x-ray detectors has revolutionized macromolecular crystallography for the collection of diffraction data. The very high synchrotron source flux densities (photons per mm^2) allow weakly diffracting or smaller crystals to be used for structure determination. They provide parallel and stable beams, many of which can be tuned to deliver incident x-ray energies from 6 keV to 20 keV (~ 2.1 to 0.62 Å), giving access to the absorption edges of a wide range of metals for experimental phasing by the MAD method. Pioneering beamlines suitable for data collection at longer wavelengths (up to 4 Å) are under construction to enable more experimental phasing of structures using the anomalous signal from intrinsic sulfur atoms in proteins. The now robust top-up mode at synchrotron sources, in which the storage ring is continuously fed with electrons, results in stable experimental conditions for long periods of time. Detector technology has moved on apace, driven by the requirement for faster and larger position-sensitive devices. Originally, the field used photographic film and proportional counters, and then position-sensitive multiwire gas-filled detectors, adapted television tubes, imaging plates (reusable film), charge-coupled device detectors, and, most recently, pixel detectors (8).

Most synchrotron beamlines are currently equipped with sample-mounting robots that transfer crystals from a liquid nitrogen Dewar to the goniometer into a stream of 100 K nitrogen gas, meanwhile keeping them cryocooled. The increased reliability of these robots has led to remote data collection in which crystals are delivered to the beamline and the researcher controls the beamline hardware remotely. Synchrotron beamline availability is now such that many in-house systems are being decommissioned.

A number of synchrotron beamlines are now providing particular special facilities, such as microfocus beams (diameters down to 1 μm). With the necessary supporting software, these beams can be used to map the diffraction properties of a crystal so that the

best place for data collection can be selected. To minimize background and maximize the signal-to-noise ratio, the beam and crystal size should be matched. Thus, these microbeams are ideal for use with microcrystals, where many crystals can be mounted on one loop and then individually irradiated.

Additional instruments have been made available to augment the information that can be obtained from crystals through simultaneous data collection using complementary techniques. For example, most synchrotrons now have a beamline onto which a microspectrophotometer can be mounted, which can provide valuable data on redox protein states and radical formation during x-ray irradiation (9). Another useful new addition is a device to carry out on-line controlled dehydration of protein crystals (10), because in some cases this technique can improve the diffraction quality in a reproducible way. For instance, F1 adenosine triphosphatase crystals were improved from 6.0 Å to 3.84 Å resolution by dehydration (10).

Automated data reduction pipelines are now widely available at most beamlines, and these allow on-line evaluation of the results so that more data can be collected immediately if necessary, substantially improving the outcomes of the experiment. However, even for cryocooled crystals, the age-old problem of radiation damage remains an issue and can result in failed structure solution due to the degradation of diffraction quality and the onset of specific structural damage (11) before enough data have been obtained. Research is ongoing to understand the variables involved and to seek mitigation strategies (12). The extent of damage at cryotemperatures is proportional to the absorbed dose, and an experimental dose limit of 30 Mg, beyond which structural information may

become compromised, has been determined (13). Software (Raddose-3D) is available to model 3D dose profiles for a range of experimental strategies (standard, helical, and translational). These simulations can be used to plan experiments that result in more homogeneous dose distributions, reducing the extent of differential radiation damage across the sample and improving data quality (14).

A number of streamlined packages are available to analyze the diffraction data and to reduce them to a unique set of reflections so that structure solution can commence. Concomitant with the developments in hardware and the automation of data collection, computational tools for structure solution have seen huge progress over the past decade. Crystallographic software, such as that distributed by Collaborative Computational Project Number 4 (CCP4) (15) and PHENIX (16), can now solve many structures without human intervention, from data reduction through phasing and electron density map calculation, map interpretation (model building), structure refinement (completion), and deposition in the Protein Data Bank (PDB). For the cases in which automated solution is still not possible, the software is better able to analyze the pathologies causing it to fail and to guide the crystallographer to a manual solution. Molecular replacement can now succeed with very distant models or even secondary structure elements, as implemented in Phaser (17) and Arcimboldo (18). Experimental phasing can now succeed with very weak anomalous signals due to progress in phasing software [e.g., the SHELX suite (19)] and improved methods to enhance the anomalous signal when combining data collected from a large number of different crystals [e.g., (20)].

After an initial model is obtained, the structure must be refined to optimally match the model to the electron density. This process is fast and has a wide radius of convergence—for example, in Phenix.refine (16) and Refmac (21). Software for automatically building atomic models into electron density maps is increasingly more robust, and for manual building, programs such as Coot (22) tremendously aid the iterative process of model refinement and rebuilding. The graphical capability now available allows macromolecules to be represented much more speedily, cheaply, and conveniently than with balsa wood and wire models (Fig. 1, A and B). For the last step in the pipeline, convenient new tools are also available for the validation of the geometry and quality of structures before submission of atomic coordinates to the PDB (23).

Future Growth Areas

Current growth areas in which macromolecular crystallography is likely to have considerable future impact include membrane protein structure solution, renewed interest in room-temperature structure

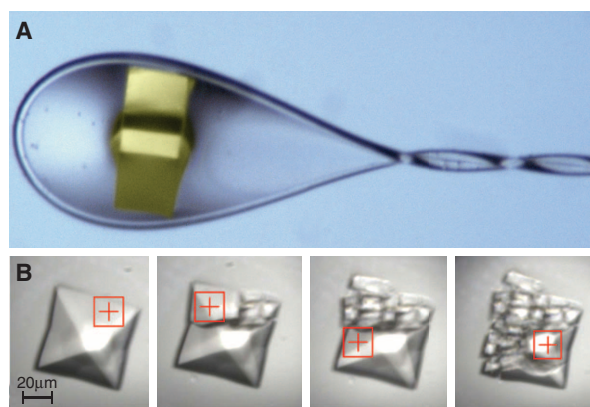


Fig. 3. Macromolecular crystals ready for data collection. (A) Cryocooled 0.5-mm-sized crystal of *Salmonella typhimurium* neuraminidase in a 20- μm -thick rayon fiber cryoloop held in a 100 K nitrogen gas stream. The transparent film of solid cryobuffer supporting the crystal indicates that no crystalline ice has formed that could interfere with the crystal diffraction pattern. **(B)** In situ data collection from bovine enterovirus crystals; despite the rapid and dramatic disruption of the crystal lattice, small amounts of high-quality data can be collected in a serial manner until a complete data set is obtained (30). Reproduced by permission of the International Union of Crystallography (IUCr).

determination at synchrotrons, and the possibilities offered by XFEL x-ray sources.

About 30% of the proteins coded by the human genome are membrane proteins. Determining the structure of these represents a major challenge for conventional techniques, because the crystallization step usually relies on controlled dehydration of a solution of protein. Because proteins extracted from the membrane are by their very nature insoluble in aqueous systems, new methods have to be employed to obtain crystals; the proteins must normally be solubilized in detergents, both throughout purification from cell lysates and during crystallization. This greatly increases the number of variable crystallization parameters to be explored and makes the search for suitable conditions both time-consuming and expensive. The addition of detergents is prone to destabilize the protein, and much trial and error is required for successful outcomes. As a result, out of 97,362 protein structures (as at 28 January 2014) deposited in the PDB, there are only 1394 membrane protein structures (24), although the number is increasing rapidly. In part this is due to the development and success of a new crystal-growing technology: the “in meso” method, which makes use of lipidic mesophases and is also referred to as the lipid cubic phase (LCP) method. This uses monoolein, which has a well-characterized phase diagram of composition (water/lipid) against temperature (25). Crystallization robots to dispense LCP are now available, and they substantially simplify and accelerate the setting up of screens. However, safe removal of crystals from LCP material requires skill and patience on the part of the experimenter, so this stage is ripe for further innovation. On contact with air, the LCP can swiftly dehydrate unless additional crystallization solution is added, and it also becomes opaque and birefringent, making it hard to locate and to harvest the crystals. Once in a cryoloop and flash-cooled (no added cryoprotectant is needed) for cryodata collection, the LCP again often becomes opaque, and any crystals within it become invisible. The automated grid scans of the x-ray beam over the loop area to detect crystal diffraction above have alleviated this problem, and work to image such crystals by x-ray microradiography and microtomography is ongoing (26).

Membrane protein crystals grown in cubic and sponge phases have yielded data revealing, for example, the structural basis for the counter-

transport mechanism of a H^+/Ca^{2+} exchanger (27) and the structure of the β_2 adrenergic receptor–G protein–active complex (28), a GPCR in association with its cognate G protein. Correct functioning of GPCRs is vital for our senses of smell, taste, and sight and is also involved in almost all signaling processes, including cellular responses to neurotransmitters and hormones. Because roughly half of all modern drug targets are GPCRs, their structural elucidation is one of the major highlights of recent research.

The ability to crystallize membrane proteins in a membrane-like environment such as LCP opens the possibility of gaining more biologically relevant information on protein–lipid interactions. Such interactions help regulate subcellular localization and determine the activities of transmembrane proteins, yielding, for instance, insight into the function of the receptor tyrosine kinase family. These proteins are implicated in the progression

of many types of cancer, as well as being vital regulators of normal processes in the cell (29).

In the search for suitable crystallization conditions for membrane proteins, it is often highly instructive to test the diffraction properties of putative crystals obtained from a coarse crystallization screen. This necessity has prompted beamline scientists at a number of synchrotrons to adapt conventional goniometers so that entire 96-well crystallization plates can be mounted in the x-ray beam and translated to enable irradiation of individual wells containing putative crystals. In some cases, a limited rotation capability has also been incorporated into the beamline hardware and software, so that complete ensemble data sets constituted of images from many crystals can now be collected and can result in successful structure solution (30), without the necessity for any post-growth handling of crystals. Figure 3 shows a crystal of bovine enterovirus at room temperature in a

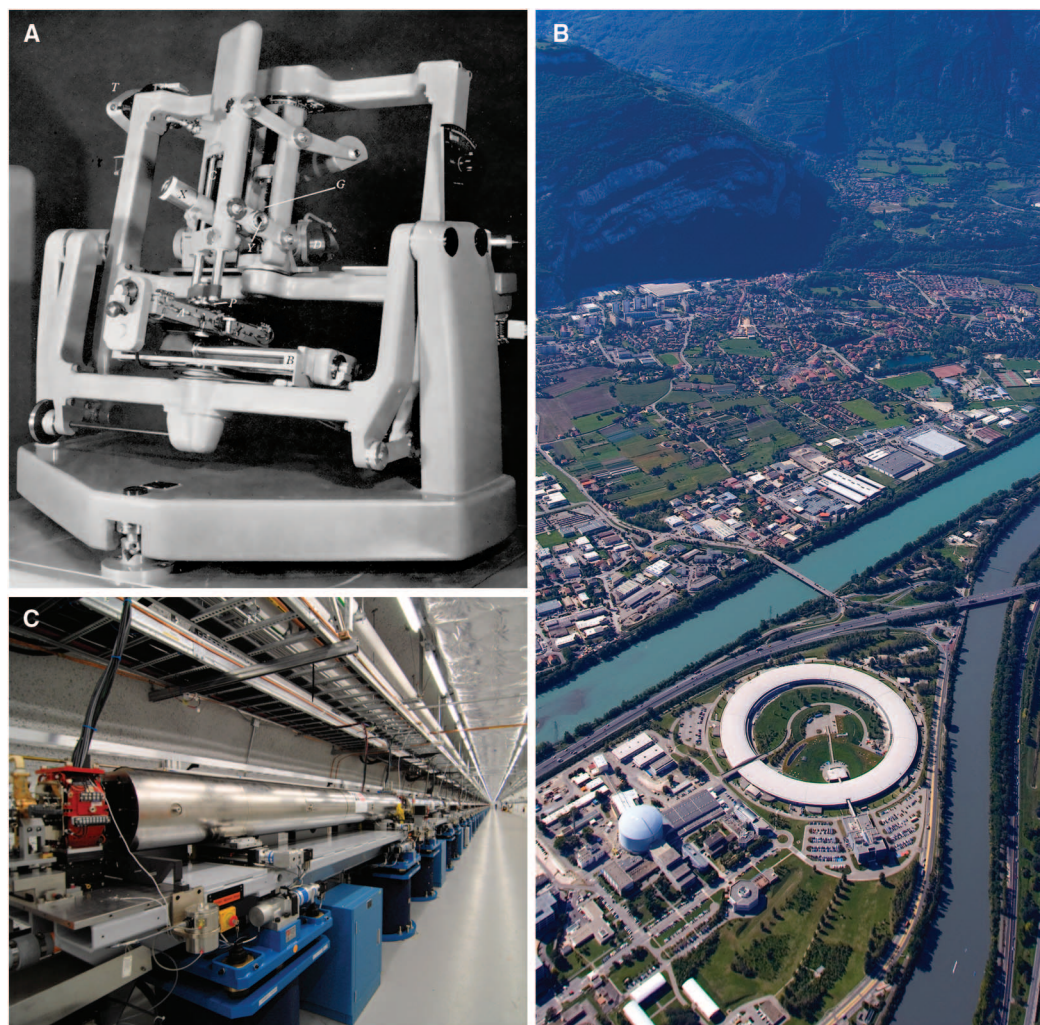


Fig. 4. Progression of hardware for macromolecular crystallography experiments. (A) A Hilger-Watts linear diffractometer as used to collect the data used to solve the structure of lysozyme in 1965 (49). (B) The first third-generation synchrotron x-ray source: the European Synchrotron Research Facility (ESRF), Grenoble, France. Photo courtesy of ESRF/Morel. (C) Part of an XFEL: a 132-m-long undulator at the Linear Coherent Light Source, Stanford, CA, USA. [Photo courtesy of SLAC National Accelerator Laboratory, Archives and History Office]

crystallization tray being consecutively irradiated for 0.5 s at four different positions by translating the tray before radiation damage effects cause the disintegration of the recently irradiated part. The success of this strategy relies heavily on the high speed of data collection and on the advent of extremely fast pixel array x-ray detectors (PADs) (31). These are replacing the charge-coupled device detectors that have been the macromolecular crystallography workhorses for the past 10 years.

Currently, the biggest PAD is 425 by 435 mm² and has a readout of 0.995 ms, a maximum frame rate of 100 per second, and 6 million pixels. The PAD readout times are so fast that they have resulted in a paradigm shift in the way the diffraction experiment is carried out, with shutterless data collection becoming the norm: It is now unnecessary to oscillate the crystal over a limited angular range (~0.1 to 1°) and then close the shutter during detector readout. This change in experimental approach combined with the high PAD frame rates dramatically increases the rate at which data can be collected, while concomitantly reducing demands on beamline components such as x-ray shutters.

Experiments using a high-speed PAD have demonstrated that it may be possible to collect data at room temperature so quickly that the catastrophic effects shown on Fig. 3B can at least partially be “outrun” (32). There was already anecdotal evidence from early macromolecular crystallography synchrotron experiments 30 years ago that room-temperature crystals lasted much longer than had been expected, and during the past 5 years there has been some debate as to the existence of a room-temperature dose-rate effect on radiation damage progression. It would be most instructive to understand the details of the radiation chemistry pathways in room-temperature protein crystals during x-ray irradiation, so that the application of recent technological developments could be optimized.

In conjunction with the in situ tray irradiation described above, the opportunity to collect more room-temperature diffraction data by collecting it faster has opened up the potential for protein structures to be determined with no postgrowth handling being necessary. This is particularly pertinent for virus crystals for which biological containment requirements complicate traditional data collection methods, but it is also important for samples that prove difficult to handle or manipulate and for those that cannot be cryocooled without serious degradation of their diffraction properties.

Hardware developments for macromolecular crystallography have not been confined to the improvement in the size and accuracy of x-ray detectors. Since the early days of sealed-tube x-ray sources, crystallographers have exploited the latest technical advances to obtain brighter beams. The huge increase in source brilliance (B) (measured in units of photons per second per mm² per milliradian per 0.1% bandwidth, here called U) available today has been achieved through steady progress that has encompassed rotating anode

x-ray generators with magnetic liquid rotary vacuum seals ($B > 10^7 U$), focusing optics fabricated from alternating graded layers of high and low-atomic number elements ($B > 10^8 U$), synchrotron-fed electron storage rings equipped with bending magnets ($B > 10^{10} U$), wigglers ($B > 10^{11} U$), and then ultimately in-vacuum undulators ($B > 10^{12} U$), and finally the recent advent of XFELs at Stanford [Linear Coherent Light Source (LCLS)] (Fig. 4), Spring8 Angstrom Compact Electron-Laser (SACLA), and Deutsches Elektronen-Synchrotron (DESY) [Free Electron Laser Hamburg (FLASH)]. For example, the macromolecular crystallography CXI (coherent x-ray imaging) beamline at the LCLS is typically operated at 10 to 120 Hz, with x-ray pulses of around 10^{12} photons in a 10- μ m focus, which can be tuned from 70 to 300 fs at energies of 4 to 10 keV ($B_{\text{peak}} > 10^{33} U$; $B_{\text{average}} > 10^{21} U$).

Serif femtosecond crystallography (SFX) is a technique in which protein nanocrystals suspended in a liquid jet are streamed using a surrounding gas jacket (33) perpendicular to the beam direction so that the x-ray pulses hit them to produce diffraction stills. These patterns are recorded on special PAD detectors (34). Typically, hundreds of thousands of images are collected, a small fraction of which show a diffraction pattern, and a small percentage of these are suitable for structure solution. The collection of one still image per nanocrystal presents a major challenge for available diffraction analysis software. In an ongoing effort, new methods (e.g., Monte Carlo integration) are being employed to extract useful information from the many terabytes of data collected during every XFEL run.

Notable SFX results so far include the structures of Cathepsin B (35) and photosystem I (36), both determined by the molecular replacement method. In another highlight, a combined spectroscopic and crystallographic study gave insights into the workings of Photosystem II (37), a large complex of transmembrane molecules (Fig. 1D), vital to photosynthesis and thus to aerobic life. In late 2013, a proof of principle de novo structure determination of soaked lysozyme nanocrystals (<1 by 1 by 2 μ m³ in volume) was achieved using the anomalous signal from a gadolinium cluster derivative (38), a serendipitous link with the beginnings of macromolecular crystallography (Fig. 1B), and technologically a far cry from the original lysozyme structure determination using a Hilger-Watts linear diffractometer (39) (Fig. 4). Thus, XFEL sources promise to provide a way in which new structures can be experimentally phased for proteins and protein complexes for which only nanocrystals can be grown.

Although the full potential of XFEL sources for structural biology has yet to be realized, they are starting to fulfill their promise of overcoming the problem of radiation damage by allowing “diffraction before destruction” during the diffraction experiment. Coupled with innovative technical developments to overcome the considerable challenges posed by the need to deliver crystals, including those grown in LCP into the path of the in vacuo XFEL sample cham-

bers, they are also providing inspiration for future experiments to advance biological discovery. In particular, given enough brightness, the XFEL potentially enables the imaging of single molecules. This capability has been shown for the 400-nm-diameter mimivirus at a resolution of 32 nm (40).

However, due to the very limited availability of XFEL beamtime, the more involved data processing pathway, and the large amount of crystalline material required, the vast majority of data collection for structural biology for the foreseeable future will be carried out at third-generation synchrotron sources, at which the pipelines for data analysis and structure solution are now well established.

The three topics summarized by no means exhaust the current activity in advancing the field of macromolecular x-ray crystallography. Other interesting areas include new methods for analyzing the electron density obtained from fragment screening experiments to aid drug discovery (41) and new synergy between purely computational approaches to structure prediction (e.g., Rosetta) and refinement of structures from diffraction data (42), which opens new avenues to address ever more challenging problems.

Conclusions

From its beginnings in 1913 with the determination of the structure of rock salt (two atoms) (43), x-ray crystallography has seen many developments that have moved it into center stage as an essential discipline contributing to a broad portfolio of scientific areas. It now has the capability to define the structures of assemblies of biological molecules with as many as 300,000 nonhydrogen atoms. Since its inception, methodological developments have driven the biological insights gained from crystallography, and they will continue to do so for the foreseeable future.

Macromolecular crystallographers have organized one of the earliest examples of a repository of “big data” that is accessible worldwide and is free for academic use. This is the Protein Data Bank, into which all 3D coordinates and the corresponding structure factors (the F_s in Fig. 2), must be deposited before publication. The field has also blazed a trail in making extensive use of statistical validation tools such as the free R value (44) and in providing well-tested, thoroughly documented, and continuously supported free software necessary for structure solution (15, 16). In these respects, macromolecular crystallography is a vanguard for other research areas to follow.

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REVIEW

Femtosecond Crystallography with Ultrabright Electrons and X-rays: Capturing Chemistry in Action

R. J. Dwayne Miller^{1,2}

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Chemistry has long been appreciated to be a race against time. One wants to create conditions to drive the desired chemistry faster than other possible reaction routes. To this objective, we have been left to imagine the relative atomic motions that lead the system through an activation or energy barrier to convert to new chemical species. This conceptualization of chemistry represents a classic thought experiment that provides the unifying language connecting the different disciplines in chemistry as well as pro-

vides the conceptual bridge between biology and chemistry. The challenge is to depict transition-state structures that are taken to be energetically at the halfway point along an assumed reaction coordinate connecting reactant and product states. This exercise is a useful pedagogical tool because it emphasizes the connection between the structure at critical transition points and barrier heights. We need this structural connection in order to properly think about means to control barrier heights and thereby the chemistry (and biology) of interest. This practice can be justified for few atom systems but is questionable for most systems of chemical interest. For a molecule of N atoms, there are on the order of $3N$ degrees of freedom or dimensions to the problem to track all possible nuclear configurations. Imagine trying to map a surface with

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“Making Molecular Movies”

To get some appreciation of the experimental challenges, consider trying to build a camera to capture atomic motions on the fly, to make a “molecular movie.” What is the shutter speed required to follow chemically relevant atomic motions? If we use the case of bond breaking, the time scale involved is the time it takes two atoms to move far enough apart so that the interatomic potential is no longer binding within $k_B T$ (where k_B is the Boltzmann constant and T is the temperature).

¹Atomically Resolved Dynamics Division, The Max Planck Institute for the Structure and Dynamics of Matter, The Hamburg Centre for Ultrafast Imaging, Luruper Chaussee 149, Hamburg 22761, Germany. ²Departments of Chemistry and Physics, University of Toronto, 80 St. George Street, Toronto M5S 1H6, Canada.



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If we take as upper limits a bond displacement of 1 Å (10^{-8} cm) and two atoms moving apart at the speed of sound (10^5 cm/s) along this coordinate, the time scale for reaching the point of no return or bond breaking would be on the order of 10^{-13} s, or 100 fs (2). This time scale should be familiar. It is associated with the typical thermal sampling time from Arrhenius theory for unimolecular reactions. There are faster nuclear motions. For example, one of the highest-frequency motions is that of the OH stretch in liquid water, with a period of 10 fs. However, this motion involves very small displacements, < 0.1 Å, which is well-approximated as motion within a harmonic potential. Chemistry, as discussed above, involves far-from-equilibrium motions (angstrom scale) that involve highly anharmonic potential energy surfaces. It is these motions that are key to understanding chemistry. The time scales of evolution along reaction coordinates depends on the specific modes involved and nature of the potential energy surface at the barrier-crossing region. For the present purposes, we can use 100 fs as the canonical shutter speed needed to capture the primary motions involved in directing chemistry.

Now, consider the lighting requirements. If we wish to capture atomic positions, we need a source with carrier wavelengths comparable with or smaller than the interatomic spacing. This requirement restricts the source to either hard x-rays or high-energy electrons. Keeping within this camera analogy, image quality always depends on having sufficient lighting. The faster the shutter speed, the brighter the source needs to be to keep the image quality for a given detection method. In terms of the machine physics, it was the source brightness that was the biggest technical challenge, as will be elaborated on below.

Last, consider the film requirements for a molecular movie camera. The film providing the image is the sample. The highest spatial resolution is generally attained by using single crystals and resolving atomic structure through diffraction or reciprocal space imaging. The exposure time of the film or sample is limited by x-ray and electron-induced damage and even more greatly limited by the damage introduced by the excitation pulses needed to trigger the chemistry of interest (*vide infra*). The sample is always the limiting factor in crystallography. However, the demands on acquiring sufficient high-quality samples for diffraction reaches a new scale in time-resolved measurements. Ideally, one would like on the order of 100 time points for sufficient dynamic range. If each observation

damages the sample under the required sampling conditions (laser excitation), the sample requirements for time-resolved crystallography become enormous relative to conventional crystallography. This challenge has been met through ingenious schemes by using aerosol/liquid jet injectors (3) and self-assembling crystallography chips (4, 5) capable of providing thousands to millions of samples or frames for making molecular movies. Nevertheless, as in all crystallography it is the sample that is most limiting with respect to resolution.

One has to also consider the background problem or image contrast within this analogy. The interconversion of matter from one form to another is a rare event involving nuclear fluctuations over a barrier separating two or more stable forms of arranging the constituent atoms. For even relatively small barriers (< 0.5 eV), at any given instant there is less than 1 in 10^8 molecules within an ensemble undergoing a thermally accessed barrier crossing. How could you ever discern the reactive motions from background?

One possibility is to trigger chemical processes by using perturbative methods. This methodology has been advanced to the limit of the fundamental time scales of chemical processes (6). It is now fairly routine to access the relevant time domain with commercially available femtosecond laser systems, with even the attosecond (10^{-18} s) time domain within reach (7). Accessing such time scales does not rely on a fast detector but

rather on stroboscopic methods in which an excitation pulse perturbs the system, and at a well-defined time delay, the system is probed. In this manner, the recording can be made with a slow detector in which each time delay represents one frame in the movie of the dynamical variables being probed. The time resolution is determined not by the detector but rather by the duration of the excitation and probe pulses. The phototrigger for the structure changes involves exciting the system from an equilibrium distribution to a new point on the many-body potential energy surface that leads to the chemistry of interest, as shown schematically in Fig. 1. One typically needs quantum yields of at least 10% to extract the nuclear motions involved in the chemistry from competing relaxation channels. At this level of perturbation, the system is generally not fully reversible, and the sample needs to be exchanged between the next excitation-probe sequence. For conventional studies of static structures using x-rays, it is usually the x-ray probe that causes accumulated sample damage. For femtosecond time-resolved studies, it is the excitation pulse that leads to sample damage. It is almost by definition: If one is interested in chemistry, the very act of triggering a chemical reaction (or other structural transitions) involving thermally stable product states will lead to an irreversible change. It is the irreversible nature of the system response that imposes such enormous requirements on source brightness and makes the problem so challenging.

The consequences of this seemingly trivial technical detail need to be fully appreciated. As a generalization, it is not good enough to have simultaneous subatomic resolution and femtosecond time resolution. Within the restricted sample constraints (limited "film footage"), the problem requires sufficient source brightness to achieve femtosecond time-resolved structural dynamics near-single-shot conditions. This level of source brightness has been achieved by using new concepts for generating femtosecond electron or x-ray pulses to serve as the stroboscopic probe. The source brightness is now sufficient to light up atomic motions via diffraction on the primary time scales dictating structural transitions (2, 8, 9).

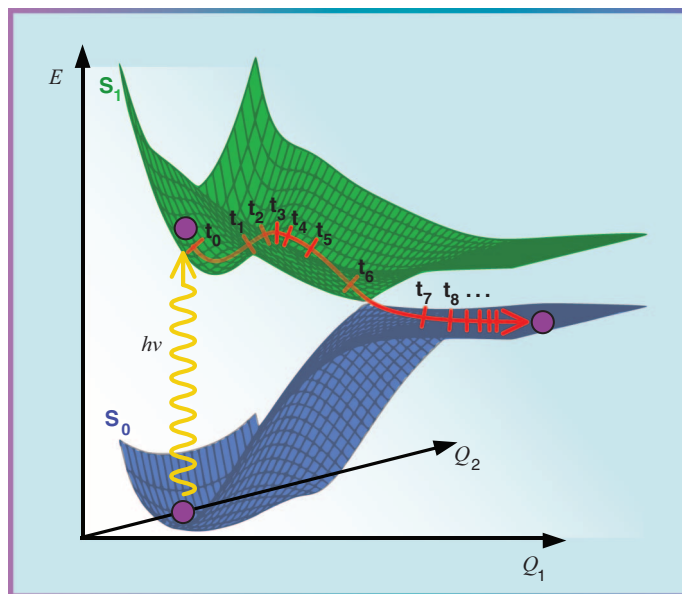


Fig. 1. Phototriggered chemistry. The basic requirement for observing far-from-equilibrium reaction modes is shown schematically. Optical excitation with femtosecond laser pulses prepares the system under barrierless conditions on an excited-state surface ($S_0 \rightarrow S_1$ transition). Even for complex molecular systems, the motions distill down to a few modes that are most strongly coupled to the reaction coordinate. Here, two heavy reduced modes are shown that are representative of an isomerization process (such as an initial step of vision) involving a bond elongation or bond weakening (Q_1) and torsional motion (Q_2). The time course is indicated as equal time steps in tracking the atomic motions to give some feel of how the ensuing motion of the atoms move in response to the potential energy gradients or reaction forces [adapted from (79)].

Evolution in Time-Resolved Crystallography

The first atomically resolved static structures were based on x-ray diffraction that date back to the days of Bragg and Laue (10, 11). Electrons for the most part have been used for real space imaging, although a number of important protein structures have been solved with electrons at various

resolution limits (12). The mean free path (coherent interaction length) depends on the scattering cross section and is dependent on composition. For low *Z* materials such as organics, the electron mean free path is on the order of 100 nm, depending on electron energy. This short coherent sampling depth limits the crystals that can be studied, although electrons are well suited for protein nanocrystallography and tomographic methods for structure determination (13). The much better match between the mean free path of x-rays and typical dimensions of high-quality crystals have made x-rays the primary source for high-resolution structure studies that use diffraction. For time-resolved studies, the x-ray-probed volume of interest is no longer defined by the crystal dimensions but by the excitation process. For the femtosecond time resolution needed to capture atomic motions, the peak power of the excitation pulses limits the samples' thickness or excited volume to micrometer dimensions to avoid excessive (terawatts per square centimeter) peak power artifacts, such as multiphoton ionization of the samples (1). There are additional considerations with electron probes to avoid blurring from multiple electron scattering. There are standard methods for sample preparation developed for electron microscopy that enable preparation of sub-micrometer samples to meet both conditions. The main difference between electron and x-ray sources reduces to spatial coherence and information content per scattering event. There are also substantial differences in infrastructure requirements as well as source stability that factor into the choice of source for a given experiment.

Electron Source Development

The major challenge in increasing the brightness of electron sources is the inherent coulomb repulsion between electrons. Brightness here is defined operationally in terms of the contrast in the diffraction observable because the spatial focus and intensity are coupled parameters. The higher the electron bunch density or intensity and the larger the transverse spatial coherence, the brighter the source. One way around coulombic repulsion issues, referred to as space charge effects, is to use very few electrons per pulse with single electron pulses being the ultimate limit to completely remove space charge broadening effects. The first experiments of chemical interest were confined to low brightness sources and gas phase systems (14–18) for rapid sample exchange. The time resolution was limited to the few-picosecond time scale by transit time differences for the electron and laser excitation pulses through the sample (19). The achieved time-resolution limit was perfect for resolving transient intermediate structures but not for determining the exact pathway through a barrier or curve-crossing region in the excited state surface. These first gas-phase experiments were pioneering studies and are still considered impressive accomplishments in low signal detection that have yet to be surpassed.

Time-resolved crystallography provides substantially higher spatial resolution and expands the problem selection to condensed phase systems. The higher spatial resolution arises from having many orders of magnitude more molecules aligned under identical conditions, thus amplifying the diffraction process and increasing the signal-to-noise ratio for structure determination. In principle, single-electron sources could be used for such studies (20, 21). However, one needs to collect on the order of 10^5 to 10^6 diffracted electrons for a reasonable diffraction pattern (22), depending on the complexity and size of the unit cell. Statistically, to ensure single-electron pulses this class of measurement would then require finding a system capable of more than 10^6 photocycles or 10^6 samples. Photoinduced chemistry in the solid state generally involves irreversible changes, as discussed above. The photoexcited volume also undergoes very slow thermal cooling of the absorbed photon energy, which further conspires to limit sampling rates to unacceptably long data acquisition times in order to avoid thermal artifacts. There is no real substitute for high brightness sources for this class of experiment. Given the inherent space charge or coulombic repulsion effects associated with electron sources, the brightness requirements seemed to be an intractable problem for electrons.

The first major advance in electron source brightness came from a numerical solution to the coupled equations of motion of some 10,000 electrons in modeling experimental conditions for electron pulse propagation in time-resolved diffraction experiments (23). These calculations showed that the space charge broadening was confined primarily to temporal broadening, and that within limits, the transverse spatial coherence could be maintained for atomic structure determination with suitable beam focus. This number of electrons is near the single-shot limit for atomic structure determination of relatively simple unit cells. Most important, the calculations showed that high bunch charge-density electron pulses do not lose space-time correlation and that nonrelativistic electrons naturally develop an extremely linear chirp during pulse broadening. Basically, electrons at the front of the pulse stay at the front, and electrons at the back stay at the back as they experience coulombic pulse broadening effects. Two means of generating electron pulses with sufficient coherence and bunch charge for single-shot imaging with atomic resolution emerged. One solution was to develop extremely compact electron guns to prevent excessive pulse broadening with propagation (8, 24). This solution required some rethinking of high-voltage feedthroughs and electron optics, but it is the simplest and most robust electron source concept for this class of experiments.

The other solution exploits the highly linear chirp and conserved spatial correlation so as to temporally compress the pulses at the sample position. There are a number of dispersive elements for electrons, similar to prisms and gratings for

optics, capable of compressing such highly linear chirped pulses with high fidelity. The most elegant solution involves the use of a radio frequency (rf) cavity to compress the pulses on axis for either shorter pulse generation (25) or higher brightness on target (26). Both the compact and rf gun concepts are capable of between 10^5 and 10^6 electrons, with effective time resolution on the order of 100 fs. The time resolution can be further improved to ~30 fs through rf pulse compression by using time stamping methods to correct for timing jitter of the rf pulse compression technology (27). Taking into account the approximately 10^6 -higher scattering cross section of electrons relative to x-rays and the need for thin samples, this source technology is comparable in both time resolution and detected particle flux with XFELs (10^{12} x-ray photons/pulse) for femtosecond time-resolved crystallography (vide infra). In this sense, these sources are ultrabright. Further increases in electron source brightness are possible with relativistic electron sources that greatly reduce pulse broadening effects (28, 29), with 10 fs time resolution within reach (1, 30). The main disadvantage of electrons is the more involved sample preparation. The clear advantage of electrons is that this source technology is tabletop and provides a very stable source for achieving high signal-to-noise femtosecond diffraction patterns.

The first grainy pictures with sufficient diffraction orders to resolve atomic motions involved in a structural transition on the prerequisite sub-picosecond (10^{-12} s) time scale are shown in Fig. 2A (8). These frames catch the simplest possible structural transition—the act of melting—but under rather special boundary conditions involved with strongly driven phase transitions. The particular question being addressed by this work dates back to a long-standing debate in the 1930s concerning the onset of the liquid state (31). This issue also has ramifications for understanding the state of matter in other extreme conditions, such as the interior of planets or stars (32). To put this question in proper context, consider the melting of a block of ice. We all know ice melts from the surface. We also know that if we direct a blow torch to the ice it will melt faster. This everyday experience is referred to as heterogeneous nucleation. What if you could heat up a material so fast that based on extrapolations of heating rates and melt velocities, you would predict that the material should melt faster than the atoms could move or, more correctly, faster than the speed of sound?

The answer can be gleaned from this data (Fig. 2A) directly, without a high level of analysis. The experiment used a special “blow torch”—in this case, a femtosecond laser system—to achieve heating rates approaching 10^{15} K/s for Al (as opposed to ice in the above analogy). At 500 fs, you can see high-order diffraction rings illustrating that the Al is still in its nascent face-centered cubic (FCC) lattice. At 1.5 ps, you can see these rings become dimmer as the initially photoexcited

electrons lose energy to lattice phonons. The increase in root mean square (RMS) motion of the atoms reduces the lattice coherence and corresponding diffraction as described by temperature-dependent Debye-Waller factors. The most astonishing event happens between 2.5 ps and 3.5 ps. There is an incredibly fast lattice collapse in which bonds are broken and the first coordination number of the FCC lattice goes from 12 to an ensemble average of 10 for the unstructured shell-like structure of a liquid. Once reaching the critical point for this degree of superheating, the whole melting process occurred within 1 ps. This time scale has to be fully appreciated. This is 10 times faster than this process could occur through normal heterogeneous nucleation. Rather than melting from the surface in an “outside-in” fashion (heterogeneous nucleation), the system was melting from the “inside-out” (homogeneous nucleation). This provided

an atomic view of homogeneous nucleation that could be used to test the accuracy of atomistic molecular dynamics calculations (33). From the real space transform, it was possible to discern that the largest changes involve shear atomic motions, with the collapse of the transverse barrier as part of forming the liquid state.

These observations showed how to control nucleation growth to as few as 10 atoms and avoid cavitation-induced shock waves and thermal damage in laser-driven ablation. On the basis of this new insight, a picosecond infrared laser tuned to the OH stretch of water in tissue was developed for laser surgery, with the correct temporal profile to restrict nucleation growth. This method has now been shown to be capable of cutting tissue without scar tissue formation (34).

Subsequent work in the area has primarily focused on photoinduced phase transitions. On the

femtosecond time scale of the photoexcitation process, the lattice is effectively frozen. The optical transition to a higher lying electronic state instantaneously changes the electron distribution relative to the time scale of nuclear motions. This provides an opportunity to observe the effects of changes in electron distribution and electron correlation energies on bonding by observing the atomic motions in response to these changes. These studies include strongly driven phase transitions involved in nonthermal melting or electronically driven nucleation effects (35, 36); creating states of warm dense matter with a counterintuitive apparent increase in bond strengths at high excitation levels (32); and the observation of highly cooperative, coherent, responses to weak perturbations of strongly correlated electron-lattice systems (37). With respect to the latter case, the highest-quality atomic movies of structural transitions have been observed

in layered compounds that exhibit interesting two-dimensional effects on electron correlation energies and bonding [(1, 37), movies]. Charge density waves (CDWs) are examples in which a small modulation of the atomic positions in the plane leads collectively to higher overall lattice stability. By photoinducing a change in charge distribution, this delicate balance in forces between intra- and interplane coupling is modified, and the lattice relaxes to a higher symmetry state. The atomic movie of the photoinduced suppression of the CDW modulation in TaSe₂ is particularly interesting; one can observe a dramatic effect in which both the suppression and reformation of the CDW occur at the fundamentally fastest possible speeds. This work has been extended to other related systems (38, 39), such as TaSe₂ (Fig. 2, B and C), in which one observes similar effects—however, with important differences owing to different inter-plane couplings. When one observes such a collective effect at the atomic level, there is an immediate appreciation of the highly cooperative nature of strongly correlated electron-lattice systems. The visual connection to the many-body effects helps to drive home the operating physics in a single measurement. Effectively, it is a direct observation of the electron-lattice coupling.

From a chemistry perspective, the real power of high-brightness electron sources has been recently demonstrated by using femtosecond crystallography to follow the photoinduced reaction dynamics of organic systems (40, 41), the

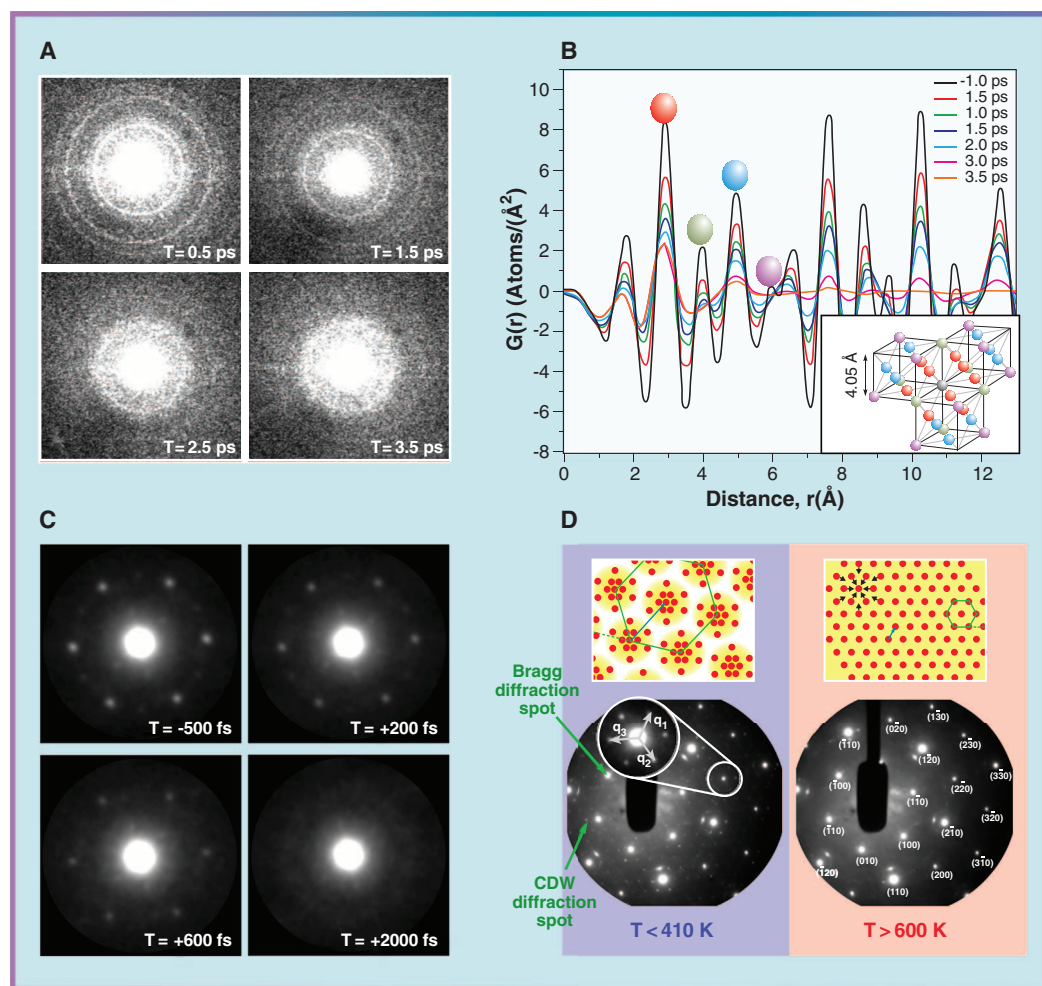


Fig. 2. Evolution in femtosecond electron diffraction. (A) The first grainy frames of an atomic movie of the melting of Al under strongly driven conditions. It is possible to see by eye the increased thermal motion leading bond breaking (0.5 to 2.5 ps) and collapse of the FCC lattice to the shell-like structure of a liquid (2.5 and 3.5 ps). (B) The real space transform (8). (C) The dramatic improvement in femtosecond electron diffraction, in which it is possible to directly observe (reciprocal space) the atomic motions involved in the suppression of CDWs in TaSe₂ for a single diffraction order out of hundreds to give the structural changes. An expanded view of the two different structures as observed in electron diffraction is shown with a schematic representation of the atomic motions captured on the 100-fs time scale (38).

mainstay of chemistry. This class of materials invariably has low thermal conductivities and involve large-amplitude motions as part of the reaction dynamics that greatly limit the sampling rate and number of photocycles. In addition, organic systems have much more complex structures than do the solid-state systems discussed above. The development of high-brightness electron sources was key to opening up this class of study. To gain some appreciation of the quality of the diffraction data that made this possible, compare Fig. 3, a weakly scattering organic system, with Fig. 2A to see the dramatic improvement with source brightness. There were hundreds of diffraction orders that went out to better than 0.4 Å to serve as constraints in the determination of the time-dependent structures. The signal-to-noise ratio of a single diffraction order is comparable with high-quality, integrated, all-optical pump-probe measurements, but with direct connection to structure [(40), figure 2, and (I), movies].

This improvement in source brightness enabled a dynamic observation of the photoinduced structural changes in the interesting charge-ordered organic system comprising ethylenedioxytetrathiafulvalene (EDO-TTF) and PF₆⁻ counterions, (EDO-TTF)₂PF₆, shown in Fig. 3. This system can be photoswitched from insulating to metallic properties (40) by means of a charge-transfer process strongly coupled to nuclear modes, stabilizing the change in charge distribution, as shown schematically in Fig. 3. Inspection of the differences between the insulating and metallic structures shows that the formation of the metallic state involves the flattening of the EDO-TTF moieties. The displacement of a bending mode toward this planar configuration would lead to an increase in the π - π wavefunction overlap between molecules and increased electronic delocalization as part of the onset to metallic properties. Within a conventional transition-state picture, one would naturally expect the bending coordinate to be the dominant mode in this process. However, this simplified line of thinking only works for few atom systems. Considering just the molecules within a single-unit cell, this problem involves over 280 different degrees of freedom or dimensions. However, it was found that all of the diffraction orders could be fit by the displacement of just three reduced modes (Fig. 3C) in which the motion of the heavy PF₆⁻ counterion appears to be the key mode. In hindsight, this observation is understandable because the photoinduced change in electron distribution will lead to a change

in the local field that will exert a force on the counterion. The PF₆⁻ ion is rather large, and its motion, through steric effects, couples the other modes. The projections along the three reaction coordinates (Fig. 3C) look like shadow projections of one another; the modes are strongly correlated.

One typically uses an approximate frozen slice of a many-body potential to discuss reaction coordinates and get a feel for the forces and types of motion involved in directing the process. However, these results show that the modes are dynamically coupled and that one cannot intuitively guess which modes are involved or the relative degree of coupling. In principle, time-dependent ab initio theory can provide the information on the relative degree of coupling between the different possible motions (*I*). There is a limit. Even the highest level of time-dependent ab initio theoretical methods have to use highly truncated model systems to approximate typical chemical reactions. In this respect, theoretical calculations of reaction coordinates are generally projected along the modes found to be most strongly coupled to the reaction coordinate. Given the level of approximations required in treating electron

correlation energies and highly simplified model structures, the observed reduction in dimensionality even within full modal basis calculations might be considered to be a consequence of the truncated moiety used to model the reaction coordinate. We now see that this approach can be experimentally justified for even very complex systems. There is in fact an enormous reduction in dimensionality that again is the key to how chemistry reduces to transferrable concepts in the form of reaction mechanisms.

The other study of an organic system with the necessary space-time resolution to directly observe the correlated atomic motions through barrier-crossing regions was that of the ring-closing reaction of diarylethene (41, 42). This study was distinct in that the process is strictly a chemical reaction and involved bond formation, as opposed to bond breaking. It is generally difficult to set up conditions without major entropic barriers to bond formation. The diarylethene system was specifically designed to give high quantum yields for cyclization to serve as an efficient photochromic material, capable of undergoing more than 10,000 photon cycling processes (43). This system would

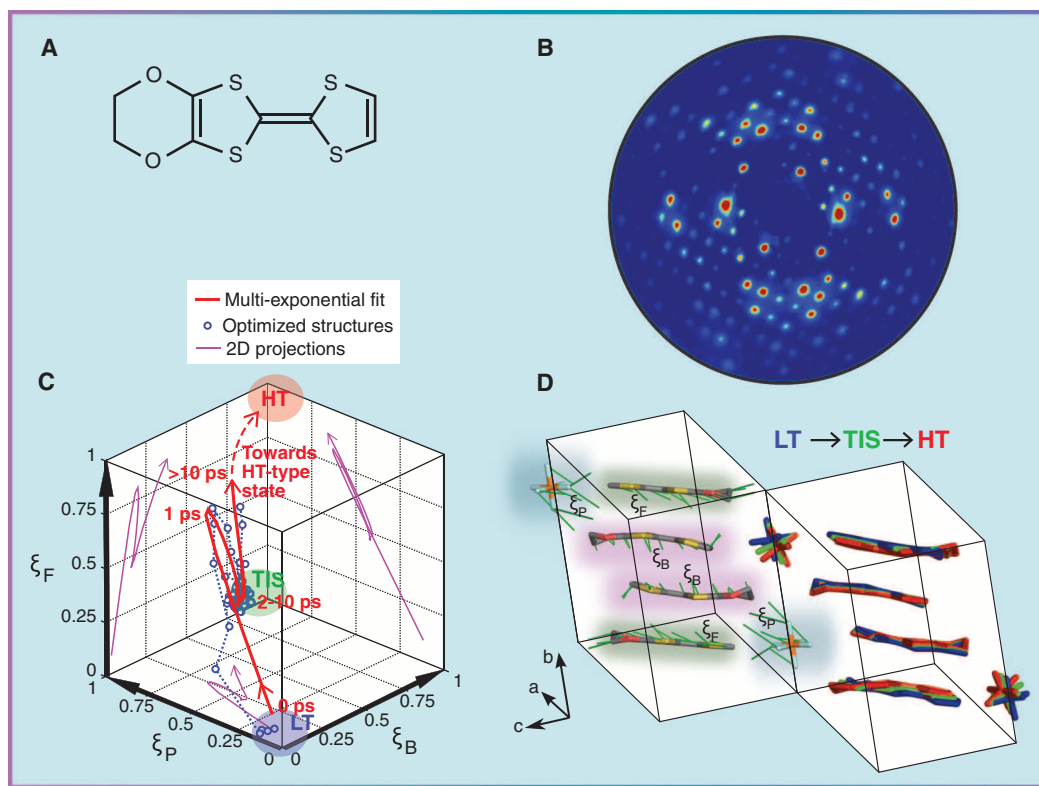


Fig. 3. Reduction in dimensionality. (A) Molecular structure of EDO-TTF. (B) Representative electron diffraction pattern to illustrate the high quality of diffraction. (C) The structural changes can be mapped onto three reduced-reaction coordinates (ξ_P , motion of the PF₆⁻ counterion; ξ_B , bending coordinate; and ξ_F , sliding motion of the rings) that stabilize the change in charge distribution, leading to electron delocalization and metallic behavior. The projections along these three normalized coordinates are highly correlated, indicating strong coupling between these nominal reaction modes. (D) Schematic depiction of the motion along these modes is given to provide a sense of the motions involved, from the insulating structure (LT), to a transient intermediate structure (TIS), to the final metallic-like structure (HT), with direction of motion indicated by the arrows and superposed structures for some sense of animation [from (40)].

appear to be an ideal candidate for even single-electron pulse probes. However, this degree of photocycling is only for low fractional excitation. At the excitation levels needed to observe the structural changes above background, even this system is only capable of ~ 100 photocycles before irreversible changes occur.

This system provides a classic example of a cyclization reaction with conserved stereochemistry. As in the case of $(\text{EDO-TTF})_2\text{PF}_6$, there is an enormous reduction in the nuclear degrees of freedom coupled to the reaction coordinate. A detailed correlation analysis of the femtosecond time-resolved diffraction patterns for the ring-closing reaction found that there is an initial motion occurring around the central bond that involves the whole molecule and brings the labile carbon atoms involved in the bond formation into close proximity (Fig. 4). With these results, it was possible to connect the actual atomic displacements that are best approximated by the lowest frequency, 55 cm^{-1} (41, 42), found in a vibrational mode analysis using density function theory. This is the key mode that directs the system to the seam in the reaction coordinate (Fig. 4A). The question is how does such a spatially delocalized mode lead to the highly localized motions needed to close the ring? Again, there is a surprise. These latter displacements leading to bond formation and ring closing occur on a picosecond time scale involving highly localized rotational motions (41). From a time-dependent *ab initio* calculation, using a truncated model system, these relaxation processes involve additional seams connecting the

product and ground state from the excited state. Experimentally, it was possible to cast out a series of localized rotational motions that mix to produce the ring-closed form. The ultrafast nature of this process clearly separates the possible modes that are involved and highlights the importance of sufficient space-time resolution to connect the initial low-frequency mode to the localized rotational coordinates. It is the mixing of modes under the highly anharmonic conditions in the barrier-crossing region that leads to the localized motions and concepts of breaking the weakest bond that chemists have empirically learned to control.

X-ray Source Development

The first time-resolved x-ray diffraction experiments to achieve picosecond to sub-picosecond time resolution were accomplished with laser-based x-ray plasma sources and Thomson scattering (44–48). The source brightness was initially insufficient to resolve more than a single rocking curve, so it was not possible to connect to structural changes. These initial studies were confined to the study of systems with well-defined, fully reversible atomic motions, such as lattice heating and impulsive excitation of lattice phonons. However, it was possible even within this limited information to provide new insights into the structural dynamics. As a case in point, it was possible to distinguish structural relaxation dynamics for the photoinduced phase transition in VO_2 (49), whereas previously it was impossible to separate the electronic and nuclear terms in transient spectra, even without full atomic details.

The next major advance in time-resolved x-ray crystallography came through the exploitation of the time structure of the circulating pulses in third-generation synchrotron light sources. These studies were capable of full atomic resolution, albeit with orders-of-magnitude-lower time resolution as a compromise. The key advance of this work over the laser plasma sources was the ability to use Laue diffraction (broader bandwidth and more information) to maximize the sampled reciprocal space (50–54). This feature in turn reduced the number of crystal orientations needed to make data acquisition for time-resolved measurements tractable. The initial studies were confined to nominally subnanosecond time resolution by the pulse duration of the electron bunch in the ring. There were also substantial challenges in coming up with new analytical methods for extracting atomically resolved transient structures from the unexcited fraction of the crystal (55). Long-lived excited states involving intersystem crossing of spin-forbidden transitions and heavy-metal centers were specifically engineered to provide model systems for testing various aspects of quantum calculations for excited states (56). The field of time-resolved crystallography quickly evolved, in which it was possible to resolve important details regarding the transient intermediate structures involved in photochemical reactions to bond displacements convolved to changes in spin crossover materials (57). These latter studies explored the quantum mechanics of how electrons change spin.

The time resolution of these synchrotron-based sources was improved by a factor of 10^3

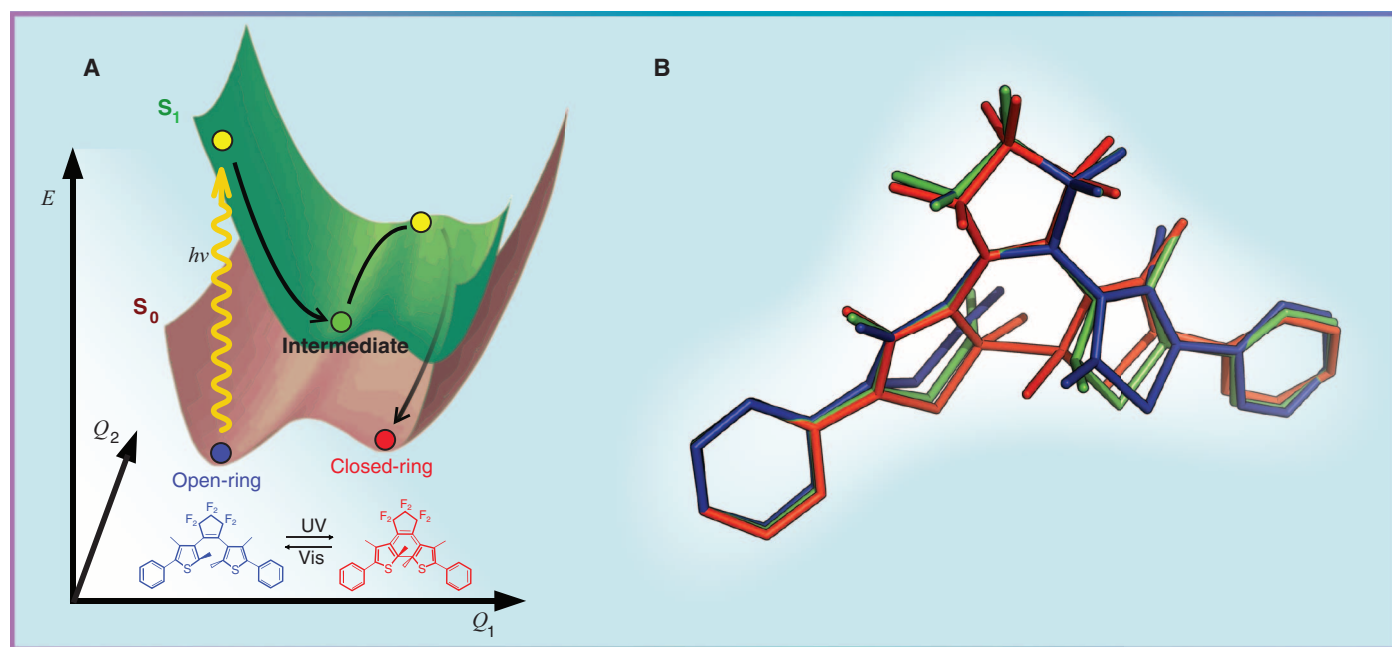


Fig. 4. Observation of the primary motions involved in bond formation and ring cyclization. (A) The structure of the specific diarylethene derivative is shown with the photocycle used to follow the ring-closing reaction. The simplified reaction coordinate is shown above for an initial bending motion (Q_1) that brings the

labile carbon atoms within wavefunction overlap, followed by localized rotation motions (Q_2) that close the ring [surface adapted from (80)]. (B) Animated view of motions recovered from femtosecond electron diffraction studies, from the initial structure (blue), to the distorted bent structure (green), to the final closed-ring structure (red).

to 10^4 —to enter the femtosecond domain—by using beam-slicing methods, in which an intense laser interaction with the electron bunch in the ring effectively cuts out a 100-fs x-ray slice from the bunch (58). There is a corresponding reduction in x-ray flux by a factor of more than 10^3 with propagation losses. These beam-slicing sources now provide very stable, relatively, broadband sources for femtosecond soft x-ray spectroscopies from which structural information can also be retrieved (59, 60). In terms of hard x-ray diffraction, the source technology is not bright enough to give more than a couple of diffraction orders for simple unit cells. This information is still sufficient to resolve the time scale for structural phase transitions, and electronically driven bond displacements have been tracked on the relevant time scales to give new insights into electronic factors involved in these effects (61).

Subsequent to this development, there has been a major advance in laser-based x-ray plasma sources. By going to very thin copper-film targets and using powder diffraction to increase the signal, it has been possible to obtain very-high-quality diffraction patterns with 100-fs time resolution (62). The use of powder diffraction to increase the number of diffraction orders was important because it also enabled the use of the background diffraction (unexcited crystal volume) of known structure to serve as a heterodyne source for signal amplification and phase retrieval. Orders-of-magnitude-fewer x-ray photons are then needed to resolve the structural changes. This approach has enabled the inversion of the time-dependent diffraction to very-high-quality electron density maps for the structural changes. These maps have been interpreted in terms of concerted electron-proton transfer in ionic crystals (62, 63), to a very interesting effect involving laser field-driven changes in electron distribution in LiH and NaBH₄ (64). There is a limitation in that the excitation process involves a nonresonant multiphoton process to uniformly excite the needed x-ray-probed volume for these studies that may lead to multiphoton effects. New advances in the drive laser promise to increase the x-ray flux by two orders of magnitude, which will correspondingly decrease data acquisition times and enable going to well-defined one-photon excitation processes for triggering the structural dynamics of interest. This approach is paving the way to the development of a versatile tabletop x-ray source for femtosecond crystallography of small-unit-cell crystals.

The major advantage of x-rays over electrons in femtosecond time-resolved diffraction experiments is in the study of biological systems. In this regard, the spatial transverse coherence of the femtosecond electron sources has not yet achieved the magnitude needed to both provide atomic resolution and be capable of studying unit cells beyond 6 nm, which is at the border of protein crystallography. New developments in photo-

cathode materials will likely solve this problem, but there are fundamental limits to electron source brightness that will limit the size of protein systems that can be studied.

The interest in time-resolved studies of biological systems was the driving force for the introduction of co-crystallized photolabile caged compounds for triggering biochemical processes (65), as well as the breakthrough in time-resolved Laue diffraction (51). To fully resolve the functionally relevant motions, the most important advance in x-ray sources has been the relatively recent introduction of the X-ray Free Electron Laser (XFEL) at hard x-ray wavelengths (3, 9). The average beam current and output power is similar to third-generation synchrotrons; however, the design principle uses compressed high-energy (10 GeV range) electron pulses to produce extraordinary gain within the undulator so that the radiated x-rays can be reduced to the few-femtosecond domain. Most important, the oscillating electron bunch radiates in phase to produce a spatially coherent x-ray beam. The high degree of transverse spatial coherence is what distinguishes this source from all other x-ray sources. The decrease in pulse duration, energy bandwidth, and increased spatial coherence correspond to an overall gain of several orders of magnitude in source brightness that can be well defined in terms of x-ray peak brilliance (photons/pulse/mm²/mrad²/1% bandwidth) (9, 10). Furthermore, the temporal duration and the number of x-ray photons per pulse are in the perfect range to provide single-shot, few-femtosecond time resolution to atomic motions (3, 9). However, femtosecond time-resolved crystallography with atomic resolution has not been achieved to date (11). This particular use of XFELs is still very much in the development stage, akin to the early use of synchrotrons for x-ray protein crystallography (66).

What are the challenges? First, the beamline involves kilometer-scale linear accelerators to get the electron bunch up to the giga-electron volt range to enter the undulator. The resultant x-ray pulses must be synchronized with the laser system used to trigger the structural dynamics within the required femtosecond time resolution or overall relative pathlength variations of less than 100 μ m on this kilometer scale. There is a time stamping tool in which a reference response function to the x-ray pulses is used to define the time origin to retrieve ~50 fs time resolution, and higher resolution is possible. The other challenge is that the x-ray gain involves effectively a traveling wave amplifier rather than a resonator, as in an optical laser. The gain is far from being depleted as it would be in a normal laser oscillator, and the initial x-ray photon amplification cascade is initiated from noise in the radiated field. The source represents a self-amplification of spontaneous emission (SASE) source. There are very large (100%) fluctuations in x-ray pulse output and substantial modulation in x-ray spectrum.

Seeding greatly reduces the amplitude noise (3) and will likely be in routine use in the near future. In addition, the spectrum is very narrow-band as compared with synchrotrons so that only a small fraction of reciprocal space is sampled, and many peaks for a given crystal orientation will be partials, meaning they are off the Bragg condition for completely constructive interference in the diffraction process (3).

These obstacles to femtosecond time-resolved crystallography have been overcome through shot-to-shot normalization, time stamping tools, high-throughput sampling, and new data analysis methods. The experiments are more demanding than conventional femtosecond laser spectroscopy. At present, these experiments require an expert user base to further develop the methodology. The most challenging problem may ultimately be the enormous number of crystal orientations and demands on sample for this class of experiment. For calibration, a typical protein crystal structure determination at a synchrotron facility requires on the order of 100 different orientations. For a narrow band source such as an XFEL, the number of required projections increases accordingly. Now consider that ideally, one would like 100 time points for sufficient dynamic range to the atomic motions. In the general case of irreversible sampling, each sampled spot is damaged by either the x-rays or by the femtosecond laser excitation to trigger the structure changes. Femtosecond time-resolved x-ray crystallography then requires a minimum basis of ~10,000 crystals or sufficiently large crystals to accommodate this large number of shots in order to provide adequately sampled reciprocal space and dynamic range to give atomic-level movies of the structural dynamics of interest. At the very least, the experiment requires two-orders-of-magnitude-higher sampling over conventional crystallography to give the time base.

The sample issues may seem to be an insurmountable problem. However, the use of large crystals and orthogonal beam geometries between the laser excitation pulse and x-ray probe pulse solves the problem of sufficient sample area and mismatch between the laser-excited volume and x-ray-probed volume (53). The time resolution is nominally sub-picosecond with this beam geometry because of transit time differences between the x-ray probe and laser excitation pulses. For highest time resolution, one would like to use near-collinear beam geometries. This geometry then requires crystal dimensions on the 10-micrometer scale or less for highest time resolution and to avoid excessive peak power conditions for the excitation. In this case, thousands of crystals are needed.

As mentioned above, one particularly ingenious solution for the use of XFELs in the study of nanocrystals involves aerosol and high-pressure liquid injectors (3). Nano- to micrometer-sized crystals are shot out of a nozzle under pressure

and hydrodynamic focusing in order to give a well-defined stream of crystals that can then be sampled by the x-ray beam downstream. This methodology has gone beyond proof-of-principle experiments with the high-resolution structure determination of the disease-causative agent in sleeping sickness that to date has only been successfully grown *in vivo* as microcrystals (67). As discussed in the accompanying article (10), the importance of the high brightness of the XFEL is that the diffraction can be attained before the onset of x-ray-induced damage so that the highest-quality diffraction is attained, limited only by the crystal quality. The use of this approach has also been recently demonstrated for time-resolved crystallography on the microsecond time scale. The timing in this case is determined by the travel time of the crystal along its flight path from the point of laser excitation to the point of the x-ray beam used for sampling the crystal structure (3, 68). Normally, one needs to collect a reference diffraction pattern (no laser excitation) to index the crystal orientation and then compare with the diffraction pattern attained with laser excitation at some fixed time delay (laser on) in order to determine the changes in diffraction intensities after all the proper normalizations. This experiment relied solely on collecting enough data, in which the excitation was high enough to excite all the molecules within the crystal.

The first experiment focused on the important question of the transient structures of the photo-

system 1(PS-1)/ferredoxin system involved in the photoreduction of ferredoxin as part of the solar energy transduction processes in plants. Structural changes were observed on the 5- to 10-microsecond time scale based on the change in amplitudes and baseline of what is an effective powder diffraction pattern [(68), figure 3D]. There were not enough diffraction patterns that could be indexed to invert to structure. Nevertheless, this result illustrates that long-time dynamics can be obtained this way, in which long laser excitation pulses can be used to excite 100% of the crystal. It is not clear that this approach will be suitable for femtosecond time resolution, in which only fractional excitation is generally needed in order to avoid excessive peak powers in the laser excitation. A reference diffraction pattern is important in this limit. An alternative approach that has been recently demonstrated is to use Si nanofabrication methods to make a crystallography chip that is capable of self-assembling thousands of crystals in seconds on an array of specifically designed features so as to spatially localize the crystal and introduce random orientations (4, 5). This solution may also facilitate the use of synchrotron microfocus beam lines for high-throughput protein crystallography. Most important for time resolved studies, it enables a reference diffraction pattern to be collected. The basic principle of loading the crystallography chip was demonstrated by using fluorescently labeled polystyrene particles 2 μm in diameter [(4), figure 4]. For this size

of particle, the features of the chip can be used for size exclusion, and the density of the chip can approach 1 M-crystal pixels/ cm^2 with 75% fill factors. This concept uses the least amount of material possible, which is a critical consideration for precious protein crystals. There are a number of experiments in the pipeline that use different sample delivery systems, and one can expect groundbreaking experiments to be reported in the coming year.

The extension of femtosecond time-resolved crystallography to the study of protein dynamics will be an important development. In this regard, proteins have evolved to control barrier heights and thereby optimally control the transduction of stored chemical potential into functions. It is the passage over the barrier (on 100-fs time scales) that inextricably links chemistry to biology. In chemical terms, this problem becomes extremely interesting in terms of scaling chemistry to the next length scale of molecular synthesis. Taking into consideration the enormous reduction in dimensionality that occurs in relatively simple molecular systems, how can we make any sense of biological systems? The number of nuclear degrees of freedom for one of the simplest biological systems that serves as our benchmark for molecular cooperativity is the binding of oxygen to heme proteins (Fig. 5). This problem involves literally thousands of degrees of freedom. The chemistry aspects occur at the binding site. Here, one can define the

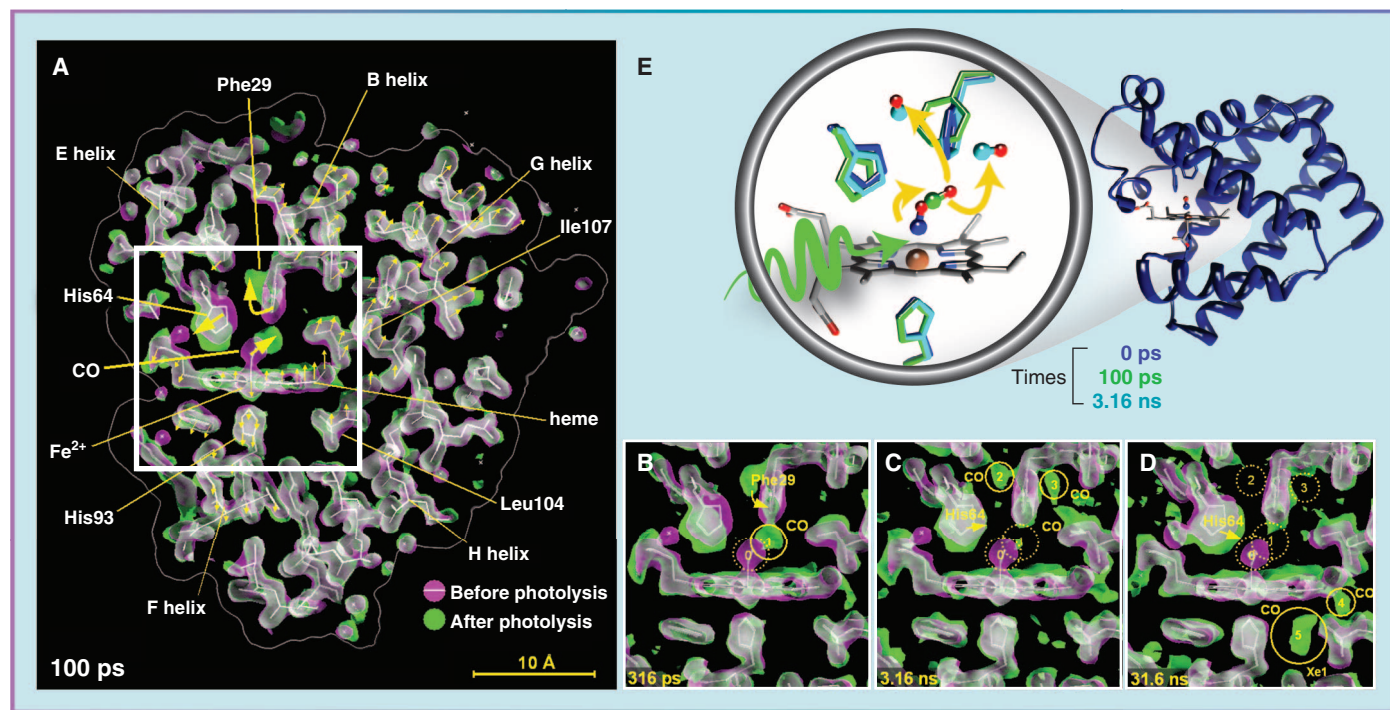


Fig. 5. Chemistry driving functionally relevant protein motions. (A to D) The structural changes following photodissociation of the CO ligand of carboxymyoglobin are shown at various time points, as indicated by the color gradients in the electron density maps [from (53)]. These motions are shown

schematically in (E) using the corresponding protein data bank files 2G0V (100 ps) and 2G10 (3.16 ns). The earliest time point at 100 ps illustrates that the CO ligand has moved 2 Å from its binding site after bond breaking, along with substantial protein motions not resolved within this time resolution.

active site as the chemical system and the surrounding protein as the bath. The big questions surround the coupling between the chemistry at the active site and the surrounding protein that leads to changes in protein structure, which cascades into molecular feedback control of coupled chemical reactions and biological functions (69–71). On the basis of the observed time scales, biological systems clearly course grain sample their potential energy surface to direct chemical energy into functions (72). Nature has highly optimized the system-bath coupling to take advantage of the inherent correlations imposed on the evolved structures. In this regard, the importance of collective modes in describing stochastic fluctuations of proteins have long been identified (73–76). There is also experimental evidence that has led to the collective mode-coupling model to explain the relationship between the chemistry and the functionally relevant motions encoded in protein structure (69, 70, 77). These anharmonic motions are highly damped to overdamped relaxation modes and are not amenable to spectroscopic investigation. The direct observation of the highly correlated motions involved in biological response functions will give us our most fundamental (atomic-) level basis to understand the structure-function correlation in biological systems.

How close are we to this goal? Shown in Fig. 5 is the high degree of information available with full atomic resolution in catching protein structural changes. This particular study (53) is representative of a number of related studies (50, 51, 78). The time resolution in following the dynamics of CO dissociation in myoglobin in this case was 150 ps (53). One can clearly see highly localized changes involved in the ligand dissociation and spatial transport out of the protein. The motions appear to be localized, but there is a high degree of steric coupling between the protein fluctuations and motion of even a simple diatomic molecule. The structural changes must involve correlated motions over some unknown length and time scales. Imagine if we could watch the chemistry unfold at the active site and its coupling to the protein motions on the 100-fs time scale to catch these details. We are getting close.

Summary and Future Outlook

The technical challenges posed for the development of ultrabright electron and x-ray sources for the observation of atomic motions have been met. As we enter this new age of atom gazing, we have already been able to see the enormous reduction in dimensionality in barrier-crossing regions that makes chemical concepts transferrable from one molecule to another. We now have the tools to directly observe the far-from-equilibrium motions that lead to chemistry. Each class of chemical reaction will have a distinct power spectrum related to the key modes that most strongly couple to the reaction coordinate. It is early days, but it may be possible to one day categorize these far-from-

equilibrium reaction modes in much the same way that we discuss normal modes in vibrational spectroscopy in relation to equilibrium fluctuations. These new advances will provide the benchmarks for driving progress in time-dependent ab initio theoretical methods in order to understand chemistry on a grander level. Additionally, these new insights will equally improve our understanding of the structure-function relationship in biological systems and ultimately may lead to a systematic basis for categorizing protein structural motifs in terms of controlling the system bath coupling—coupling between active sites and the surrounding protein. At this point, we will be able to connect structure to dynamics in improving rational control of chemistry from synthesis to drug design.

The primary events of chemistry can now be studied at the atomic level of inspection over the relevant length and time scales, and an atomic-level basis for understanding the connection between chemistry and biology is in sight. Improvements in source brightness will continue as needed. The real challenge is the development of systems that can be optically triggered to probe different aspects of chemistry and biology.

Crystallography brought us our first atomic pictures of matter. With brighter sources, it is now enabling the connection between structure and dynamics in how things work at the atomic level of detail. Not being able to resist the pun, the future of femtosecond crystallography is bright indeed.

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Dynamic Reorganization of River Basins

Sean D. Willett,* Scott W. McCoy, J. Taylor Perron, Liran Goren, Chia-Yu Chen

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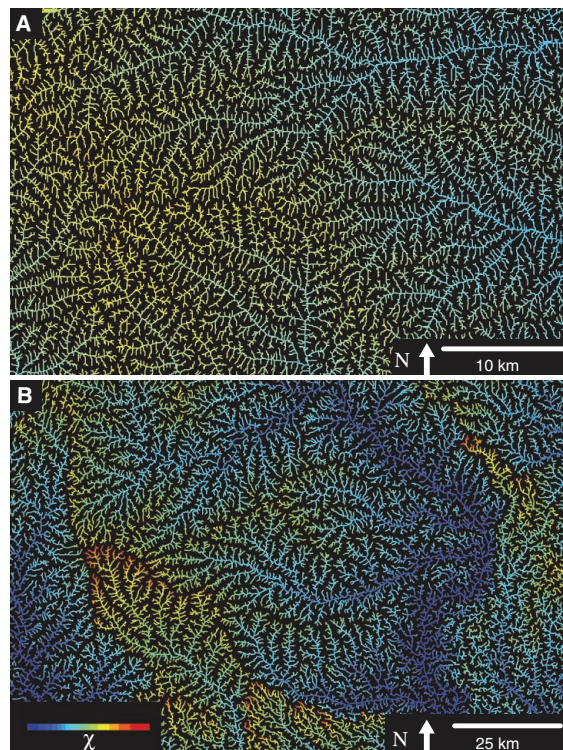
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Introduction: River networks, the backbone of most landscapes on Earth, collect and transport water, sediment, organic matter, and nutrients from upland mountain regions to the oceans. Dynamic aspects of these networks include channels that shift laterally or expand upstream, ridges that migrate across Earth's surface, and river capture events whereby flow from one branch of the network is rerouted in a new direction. These processes result in a constantly changing map of the network with implications for mass transport and the geographic connectivity between species or ecosystems. Ultimately, this dynamic system strives to establish equilibrium between tectonic uplift and river erosion. Determining whether or not a river network is in equilibrium, and, if not, what changes are required to bring it to equilibrium, will help us understand the processes underlying landscape evolution and the implications for river ecosystems.

Methods: We developed the use of a proxy, referred to as χ , for steady-state river channel elevation. This proxy is based on the current geometry of the river network and provides a snapshot of the dynamic state of river basins. Geometric equilibrium in planform requires that a network map of χ exhibit equal values across all water divides (the ridges separating river basins). Disequilibrium river networks adjust their drainage area through divide migration (geometric change) or river capture (topologic change) until this condition is met. We constructed a numerical model to demonstrate that this is a fundamental characteristic of a stable river network. We applied this principle to natural landscapes using digital elevation models to calculate χ for three, very different, systems: the Loess Plateau in China, the eastern Central Range of Taiwan, and the southeastern United States.

Results: The Loess Plateau is close to geometric equilibrium, with χ exhibiting nearly equal values across water divides. By contrast, the young and tectonically active Taiwan mountain belt is not in equilibrium, with numerous examples of actively migrating water divides and river network reorganization. The southeastern United States also appears to be far from equilibrium, with the Blue Ridge escarpment migrating to the northwest and the coastal plain rivers reorganizing in response to this change in boundary geometry. Major reorganization events, such as the capture of the headwaters of the Apalachicola River by the Savannah River, are readily identifiable in our maps.

Discussion: Disequilibrium conditions in a river network imply greater variation of weathering, soil production, and erosion rates. Disequilibrium also implies more frequent river capture with implications for exchange of aquatic species and genetic diversification. Transient conditions in river basins are often interpreted in terms of tectonic perturbation, but our results show that river basin reorganization can occur even in tectonically quiescent regions such as the southeastern United States.



FIGURES IN THE FULL ARTICLE

Fig. 1. River basins and river profiles in equilibrium and disequilibrium.

Fig. 2. Effect of drainage area change on χ .

Fig. 3. Numerical model of drainage divide migration.

Fig. 4. Map of χ for part of the Loess Plateau, China.

Fig. 5. Map and perspective views of χ for part of the eastern Central Range, Taiwan.

Fig. 6. Map of χ in river basins of the southeastern United States.

Fig. 7. The Savannah and Apalachicola river capture.

Fig. 8. Disequilibrium basins of the North Carolina coastal plain.

SUPPLEMENTARY MATERIALS

Figs. S1 to S15
Tables S1 and S2
Movie S1
Databases S1 to S9

Maps of χ for two river networks. (A) Part of the Loess Plateau, China. The values of χ are nearly equal across drainage divides at all scales, indicating that the river is in topologic and geometric equilibrium. Map is centered on 37°4' N 109°35' E. (B) Part of the coastal plain of North Carolina, southeastern United States. Large discontinuities in χ across divides indicate that the network is not in geometric equilibrium. Water divides generally move in the direction of higher χ to achieve equilibrium, so subbasins with prominent high values of χ are inferred to be shrinking and will eventually disappear. Map is centered on 35°10' N 79°8' W.

The list of author affiliations is available in the full article online.

*Corresponding author. E-mail: swillett@erdw.ethz.ch

Dynamic Reorganization of River Basins

Sean D. Willett,^{1*} Scott W. McCoy,^{1,2†} J. Taylor Perron,² Liran Goren,^{1‡} Chia-Yu Chen¹

River networks evolve as migrating drainage divides reshape river basins and change network topology by capture of river channels. We demonstrate that a characteristic metric of river network geometry gauges the horizontal motion of drainage divides. Assessing this metric throughout a landscape maps the dynamic states of entire river networks, revealing diverse conditions: Drainage divides in the Loess Plateau of China appear stationary; the young topography of Taiwan has migrating divides driving adjustment of major basins; and rivers draining the ancient landscape of the southeastern United States are reorganizing in response to escarpment retreat and coastal advance. The ability to measure the dynamic reorganization of river basins presents opportunities to examine landscape-scale interactions among tectonics, erosion, and ecology.

The recognition that Earth's surface topography evolves over time (1) provides a basis for reconstructing past tectonic (2, 3) or climatic processes (4, 5), as well as the formation of sedimentary sequences. The creation of geographic barriers or connections as topography evolves also influences speciation (6, 7) and biodiversity (8). Despite the importance of landscape change and the long history of research, establishing rates of change, or even determining whether a given landscape is in a steady or disequilibrium state, has proven remarkably difficult. Geochronological techniques have made it possible to measure long-term rates of erosion (9, 10), but large spatial and diverse temporal scales make it very challenging to assess topographic change by measuring erosion rates throughout an entire landscape.

The evolution of topography is fundamentally coupled to changes in river channel networks, which carve the landscape into ridges and valleys as they erode through rock. River basins are part of a dynamic system in which channel geometry, channel gradient, and network topology adjust toward a balance between tectonic uplift and erosion (11), a balance that depends on climatic conditions and the erodibility of the rock substrate (12). Examples of major changes in the topology or geometry of river networks have been reported on the basis of irregular network shape (13, 14) or geologic evidence such as sediment provenance (15), and geologists have described these events with marked terms like river capture and stream piracy (13, 16). However, without a straightforward way to map the motion of individual drainage divides, let alone the adjustment of an entire landscape, most studies of river systems

have focused on the equilibration of vertical incision rates while assuming that map-view basin geometry remains fixed in time (2, 3).

Recent efforts to generalize the long-term evolution of river networks have found support for drainage basin reorganization. Theoretical (17), experimental (18), and modeling studies (19–22) have argued that water divides are dynamic features of a landscape that routinely migrate, either progressively or through discrete river capture, in some cases even leading to complete reorganization of river networks (17). However, this theoretical progress has not been matched by observational support, which has remained limited to interpretations of large-scale capture events where geologic and geomorphic evidence is available (14, 23, 24).

We incorporate this view of mobile drainage divides into a procedure for mapping the dynamic evolution of river networks. We posit that equilibrium in a landscape requires that river network topology and drainage basin geometry adjust to maintain stationary water divides. This principle leads to a quantitative criterion for landscape disequilibrium that provides a prediction of drainage divide migration and a tool to map past, present, and future changes in a river network. We demonstrate this technique through analysis of a numerical model of landscape evolution and then apply it to several study sites, mapping transient features associated with river network reorganization.

A Measure of River Basin Disequilibrium

Most landscapes comprise river channels that erode bedrock and transport sediment out of the system and hillslopes that erode by a variety of processes but remain in long-term equilibrium with their bounding river channels (25). The map-view arrangement of river basins evolves through the horizontal motion of drainage divides, which is a consequence of differential rates of river channel erosion on opposite sides of the divides. A measure of the disequilibrium of opposing river channels can therefore be used to infer the stability of the intervening divide and the direction of divide motion that would bring the adjoining channels toward equilibrium.

In a river network, two flow paths that originate at a common drainage divide and terminate at a common base level experience the same drop in elevation, but their steady-state elevation profiles—the theoretical profiles for which erosion would balance rock uplift—may differ depending on the topologic and geometric structure of the network. The uppermost parts of the flow paths, between the drainage divide and the channel heads where the river system initiates, traverse hillslopes. However, several factors imply that hillslopes do not contribute substantially to differences in the flow paths' steady-state elevation profiles. First, the elevation drop along hillslopes is typically small relative to that in river channels. Second, steady-state hillslope relief is about equal on either side of the divide: Equilibrium hillslopes have similar gradients (26), and channel heads tend to be equidistant from divides (25). We therefore limit our analysis to the river channels. If drainage basins on opposite sides of a divide are structured such that the steady-state elevation of one channel head is higher than that of its opposing channel, the divide is not stable, and the network must adjust to bring the divide to a stable position. In general, any change in network topology or geometry that brings the cross-divide difference of steady-state channel head elevation to zero will bring the system into equilibrium. The simplest way to achieve this is by divide migration toward the basin with the higher steady-state elevation. Our strategy is to determine steady-state channel elevations and, thereby, directions and patterns of divide motion that allow the landscape to attain an equilibrium for both channel elevations and divide positions.

Steady-state channel elevation can be estimated from a model for river incision into bedrock. The most common model is based on the hypothesis that incision rate is proportional to the rate at which the flow expends energy as it travels over the bed, or stream power (27). Stream power depends on channel slope and river discharge, although the latter is generally replaced by the upstream drainage area, A , which serves as a readily available proxy. Including rock uplift due to tectonics, U , the elevation of a point in a channel, z , varies with time, t , and distance along a channel, x , according to

$$\frac{\partial z(x,t)}{\partial t} = U - KA^m \left| \frac{\partial z(x,t)}{\partial x} \right|^n \quad (1)$$

where K is a constant that depends on rock erodibility, precipitation rate (28), and channel geometry, and m and n are empirical constants. Other physical models, for example, based on bed shear stress, lead to an identical form of the erosion law; only the parameters, in particular, the exponents, are different. In most cases, these are determined empirically (see Materials and Methods), so Eq. 1 can be regarded as general for any erosion law that incorporates power law scaling between channel slope and drainage area at steady state.

¹Geological Institute, Swiss Federal Institute of Technology, 8092 Zurich, Switzerland. ²Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*Corresponding author. E-mail: swillett@erdw.ethz.ch

†Present address: Department of Geological Sciences and Engineering, University of Nevada, Reno, NV 89557, USA.

‡Present address: Geological and Environmental Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel.

For the simple case of U and K constant in space and time, the steady-state solution of Eq. 1 is

$$z(x) = z_b + \left(\frac{U}{KA_0^n} \right)^{\frac{1}{n}} \chi \quad (2)$$

where z_b is the elevation at the river network's base level at $x = x_b$. The quantity χ is an integral function of position in the channel network (29),

$$\chi = \int_{x_b}^x \left(\frac{A_0}{A(x')} \right)^{\frac{m}{n}} dx' \quad (3)$$

where A_0 is an arbitrary scaling area, and the integration is performed upstream from base level to location x . χ is the characteristic parameter for transient solutions of the linear ($n = 1$) version of Eq. 1 (30), and it remains the fundamental scaling parameter for the nonlinear case. The inclusion of the scaling area, A_0 , gives χ dimensions of length, but the kinematic wave nature of Eq. 1 implies that χ could equally well represent a time. In particular, if $K^n A_0^m$ is included in the denominator of the integrand, χ takes on dimensions of time and, for the case of $n = 1$, it becomes the characteristic time required for a perturbation at the river's base level to reach a point x in the channel (12).

The term in parentheses in Eq. 2 represents the relative magnitudes of tectonic forcing and erosivity, and scales the magnitude of elevation. The parameter χ characterizes the river network topology and geometry, which determine how tectonic forcing generates variable topography throughout a river basin. Given the linear form of Eq. 2, it is apparent that χ serves as a metric for the steady-state elevation of a channel at location x . Thus, with constant tectonic forcing and homogeneous physical properties, a difference in χ across a divide implies disequilibrium and, presumably, motion of the divide in the direction of larger χ to achieve equilibrium (Fig. 1). This observation is the basis for our subsequent analysis: Mapping χ throughout a channel network and comparing χ values across drainage divides yield a snapshot of the dynamic reshaping of drainage basins.

Elevation- χ Scaling with Changing Drainage Area

As a divide moves, either by continuous migration or through discrete river capture, drainage area is removed from one basin and added to the other. The channel length of each affected tributary also changes, leading to a change in the steady-state elevation of each channel head bounding the moving divide, presumably moving the channels toward equilibrium as in Fig. 1. However, analysis of a simplified scenario—the effect of a sudden change in drainage area on an equilibrium elevation profile (see Materials and Methods)—illustrates a feedback between erosion rate and divide motion that complicates this system. An instantaneous change in area induces an instantaneous change in χ , throwing the affected profile into a state of disequilibrium. Figure 2 shows the

change in the χ plot (elevation against χ) of the perturbed channel for a given fractional increase or decrease of the upstream area. Area gain shifts the χ plot to the left, above the steady-state trend, and increases its length and thereby its maximum χ value, whereas area loss shifts the profile to the right, below the steady-state trend, and decreases its length and maximum χ value. A channel that lies above the steady-state trend on a χ plot erodes faster, on average, than the tectonic uplift rate (29), so a channel gaining area experiences an increase in average erosion rate, whereas a channel losing area experiences a decrease in average erosion rate. Branches of the channel network that connect to the affected channels do not necessarily experience any change in channel length, but they do experience the indirect effect of the change in erosion rate that propagates throughout the basin. This defines an important positive feedback in the system: A transfer of drainage area from one basin to another leads to changes throughout the affected drainage basins that encourage motion of the entire perimeters of the basins in the same direction as the original perturbation. The ultimate configuration of drainage divides if and when a landscape

reaches equilibrium depends on the nonlinear interactions of multiple adjacent drainage basins and cannot easily be predicted. Here, we focus only on the local direction of divide motion toward equilibrium, but we identify some situations in which the positive feedback appears to dominate.

Spatial Variations in Uplift Rate, Runoff, or Rock Erodibility

If U or K varies in space, and these variations are known, the solution for elevation can still be obtained by integration of Eq. 1. In practice, however, U and K are seldom known. It is more common to have information about relative values or spatial patterns. For example, uplift rate may vary across a fault; precipitation and runoff, which are included in K , may have a persistent spatial pattern; or rock erodibility may vary with rock type with a spatial distribution known from geologic mapping. If we express the spatial pattern of uplift and rock erodibility in terms of non-dimensional functions of space, U^* and K^* , we can bring this variability inside the definition of χ without changing its dimensionality. Defining the uplift and erodibility as $U = U_0 U^*(x)$ and

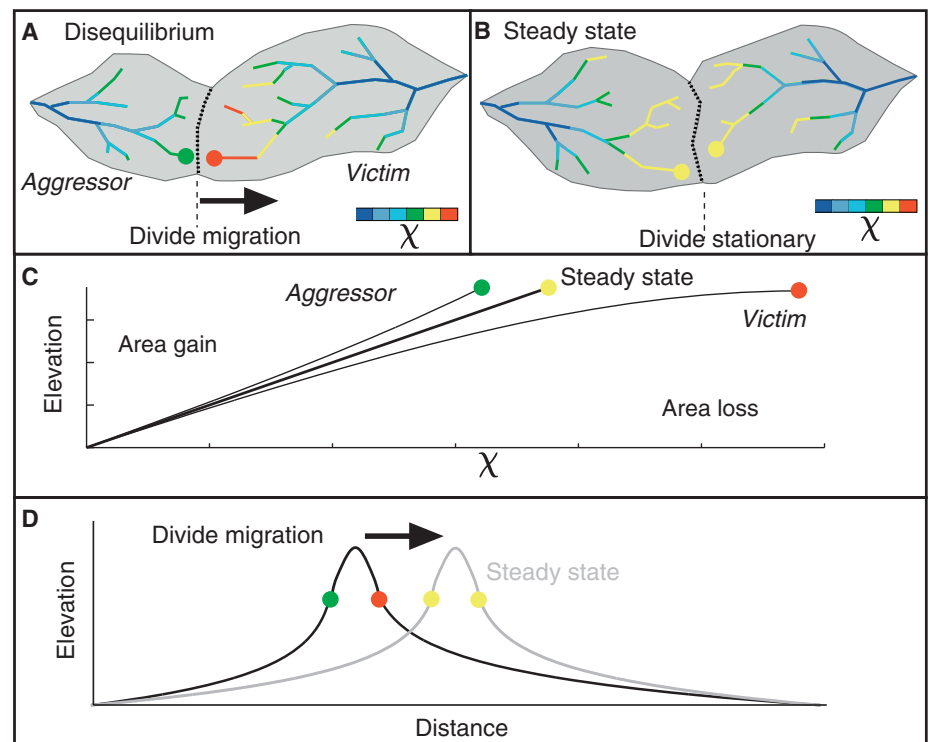


Fig. 1. River basins and river profiles in equilibrium and disequilibrium. (A and B) Change in size and shape of two drainage basins that share a common divide as they evolve from (A) a state of disequilibrium to (B) a steady state. The parameter χ (Eq. 3) provides a prediction of the steady-state elevation for a given point on a channel. The basin on the left (aggressor) has lower steady-state elevation at channel heads and therefore drives the drainage divide toward the basin on the right (victim). (C and D) The lower panels show the evolution of the elevation of two channels that meet at the shared divide with respect to (C) χ and (D) distance along the channel. The slopes above the channel head attain a symmetric form at steady state, but do not differ strongly from this form under disequilibrium conditions. The disequilibrium channel profiles in (C) show that χ is discontinuous across the drainage divide, with larger χ values in the "victim" basin. At steady state, all channel points in both basins lie on a single linear trend, subject to the assumptions described in the text. Note that changes in elevation are subtle, whereas changes in χ are marked.

$K = K_0 K^*(x)$, where U_0 and K_0 are dimensional scaling values, the steady-state elevation is given by

$$z(x) = z_b + \left(\frac{U_0}{K_0 A_0^m} \right)^{\frac{1}{n}} \chi' \quad (4)$$

with a modified χ defined as

$$\chi' = \int_{x_b}^x \left(\frac{U^*(x') A_0^m}{K^*(x') A^m(x')} \right)^{\frac{1}{n}} dx' \quad (5)$$

In landscapes with known spatial variations in U or K , Eq. 5 can be used to calculate χ' . However, if spatial variations in U and K are unknown but are likely to be minor, or if U and K vary in a systematic way over the river basins being analyzed, it may be possible to identify patterns of river basin reorganization using Eq. 3 to calculate χ , and interpreting spatial patterns of variation.

Numerical Model of Landscape Evolution and χ Evolution

To test our hypothesis that mapping of χ characterizes the transient state of an evolving river network, we conducted an experiment using a numerical landscape evolution model (27). This model solves Eq. 1 for an evolving channel network spanning a grid of nodes and calculates the positions of water divides between channels using analytical solutions for the topography of low-order channels and hillslopes (see Materials and Methods).

There are many ways that river basin reorganization can be induced in a landscape. Here,

we start with an initial steady topography generated using a linear gradient in the tectonic rock uplift rate from a lower value on the lower boundary to a higher value on the upper boundary (Fig. 3). This tectonic forcing leads to a highly asymmetrical mountain range in which the main drainage divide is closer to the upper boundary. To force river basin reorganization, we remove the uplift gradient, so that the entire domain experiences uniform uplift, and let the topography evolve until a new, symmetric steady-state mountain range develops.

The corresponding χ map (Fig. 3 and movie S1) confirms that the disequilibrium is characterized by differences in χ across divides, particularly the main divide. As predicted, divides move away from channels with low χ toward channels with high χ . In addition, we frequently observe isolated, low-order channels with high χ (Fig. 3), apparently in response to loss of drainage area and the positive feedback mechanism discussed above. These channels are transient features that tend to disappear quickly, suggesting that the area loss feedback eventually leads to a topological change in the network. Finally, we see that divides do not stabilize until χ values on either side are equal. At steady state, all points in the domain have the elevation predicted by χ , and there are no cross-divide changes in χ for adjoining channel heads. We also note that the time to steady state is many times longer than the profile equilibration time of the longest rivers in the model, demonstrating that the evolution of the network topology determines the equilibration time of the system.

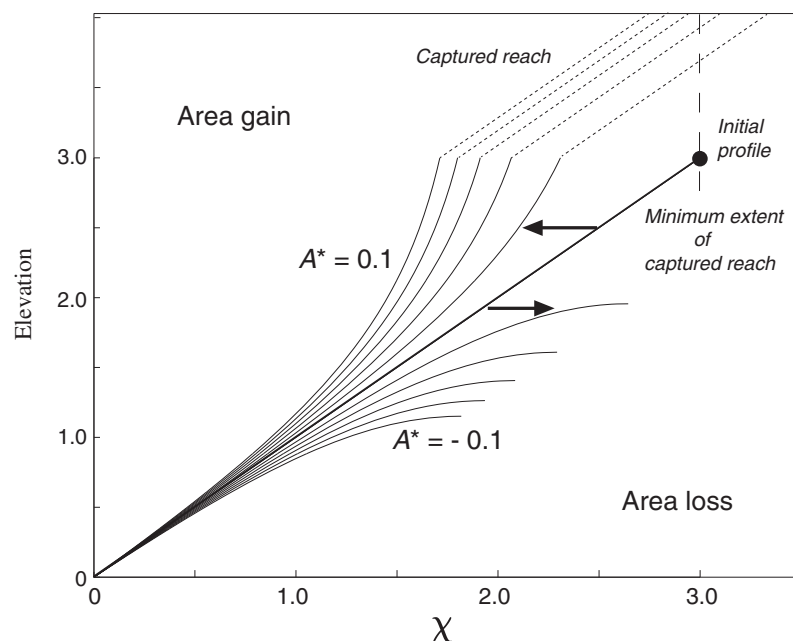


Fig. 2. Effect of drainage area change on χ . Analytical calculation of change in the χ plot in response to an instantaneous change in upstream drainage area. A^* is the applied perturbation in area divided by the initial basin area and varies from -0.1 (10% loss) to $+0.1$ (10% gain) in increments of 0.02 . Solid circle indicates the initial length of the affected channel. For area gain, the added river segment is indicated as “captured reach.” The minimum length of the captured reach length is indicated, although actual length depends on the area distribution of the captured reach. For area loss, the channel is shortened as indicated.

As this example demonstrates, mapping χ across a landscape and noting discontinuities across drainage divides should reveal not only whether that landscape is in equilibrium but also the spatial pattern of divide migration, from which we can infer a history of landscape evolution and its forcings. We investigate this through a study of a series of natural examples below.

Equilibrium Drainage Basins: China's Loess Plateau

China's Loess Plateau is a region in the Yellow River (Huang He) drainage basin where wind-blown sediments have accumulated to thicknesses of tens to hundreds of meters over the past 2.6 million years (31), draping preexisting topography (32). We constructed χ maps for two adjacent tributaries of the Yellow River, the Yanhe River and the Qingjian River (see Materials and Methods), which together drain an area of nearly 12,000 km² (Fig. 4). There are no large contrasts in χ across drainage divides at any scale, with one exception in the headwaters of the Yanhe, where a strongly curved channel has lower χ than its neighbor to the north, a sign that it is gaining area by migration in this direction. Excepting this feature, the otherwise concordant χ values across drainage divides in this landscape suggest that the channel network is nearly stationary. This river channel network was established through the Tertiary on the tectonically stable Ordos platform, and the absence of tectonic perturbation has allowed it to attain a topologic (but not necessarily topographic) steady state. This may be additionally promoted by recent incision of the relatively uniform, erodible loess, although the main features of the drainage network appear to predate deposition of the loess (32).

Basin Reorganization in an Active Mountain Range: Taiwan

The island of Taiwan was formed by collision of the Luzon volcanic arc with the Eurasian plate starting a few million years ago and continuing to the present day. Despite its youth, it has been argued that the overall height and width of the mountain range are in a steady state (33), in large part because the rates of tectonic shortening, uplift, and erosion are high (34). However, this condition does not necessarily require equilibrium at the scale of individual drainage basins. To investigate this point, we map χ in a limited area of the eastern Central Range (Fig. 5), where rock type, precipitation, and uplift rate are nearly uniform, and variations that do occur are primarily in an east-west direction, parallel to the dominant flow direction of the rivers. Rivers draining to the west of the main divide traverse different rock types and active structures with spatially variable uplift rates, so we avoid comparisons of χ across the main divide and limit our analysis to the internal and lateral divides of the eastward-draining rivers.

We find that many drainage divides at multiple scales have bounding channels with contrasting χ , which implies that the divides are unstable

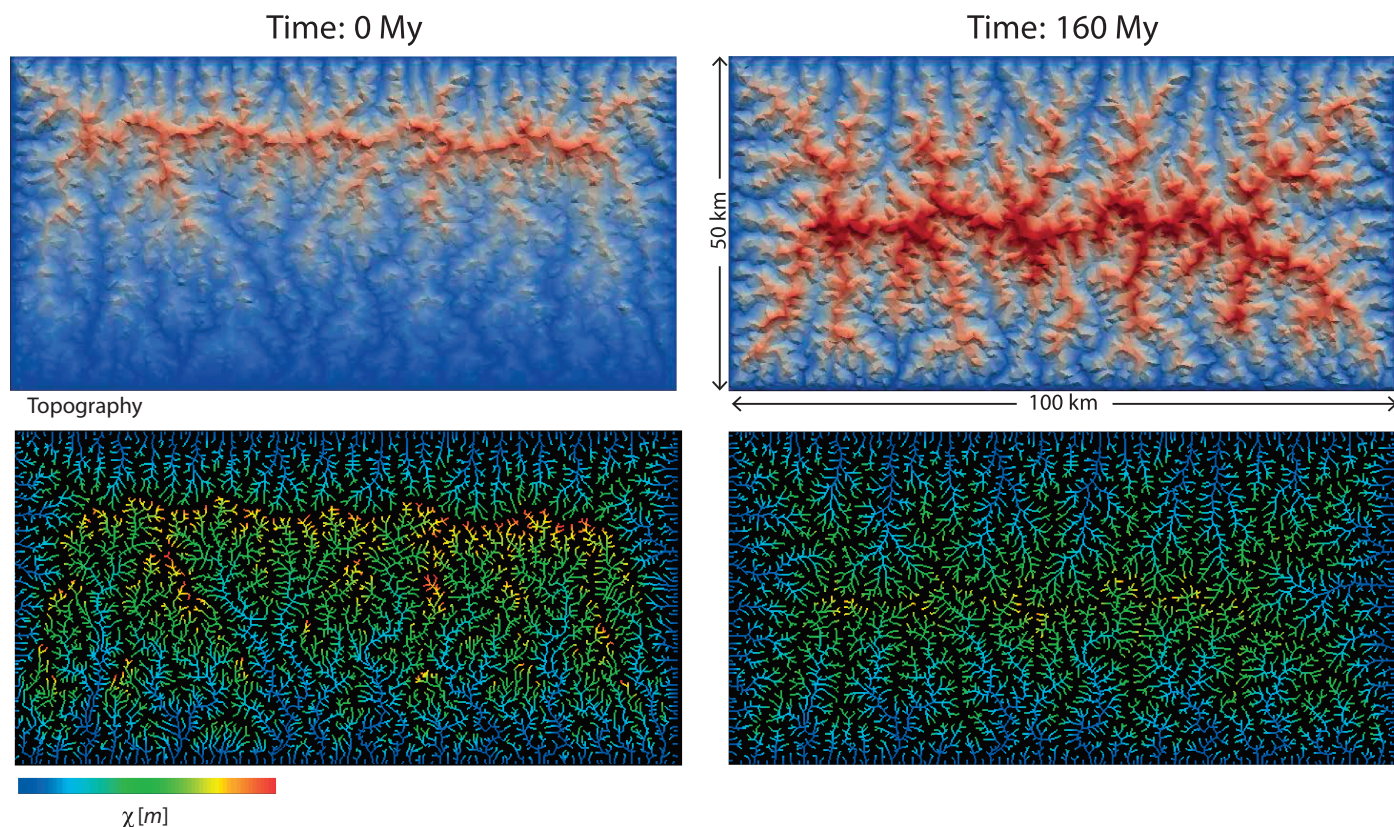


Fig. 3. Numerical model of drainage divide migration. (Left) Topography, scaled from blue (low) to red (high) (top), and χ map (bottom) of an asymmetrical mountain range generated by the landscape evolution model DAC (21) in response to a linear gradient in the tectonic rock uplift rate from 0.5 mm/year at the lower boundary to 5 mm/year at the upper boundary. The rock uplift rate is then set to a uniform value of 1 mm/year, and the landscape evolves toward a new steady state

(right) through changes in river network topology, drainage basin geometry, and channel elevations. Divide migration at multiple scales is generally in the direction of the channel with higher χ . See also movie S1. At steady state, the topography is symmetric with respect to the boundaries, and there are no discontinuities in χ across water divides. Other simulation parameters are as follows: erosivity (K) = $6.67 \times 10^{-5} \text{ year}^{-1}$, $x_c = 500 \text{ m}$, $\theta_c = 21^\circ$, $n = 1$, and $m = 0.5$.

(Fig. 5). The high relief of Taiwan allows for easy visual comparison between cross-divide contrasts in χ and topographic features visible in topographic models and satellite imagery (Fig. 5, B and C, and fig. S4). In most cases, cross-divide contrasts in χ correspond to topographic features consistent with divide migration, including lower valley elevations, steeper channels, and evidence of faster hillslope erosion in basins predicted to be aggressors.

Disequilibrium is particularly evident for several long, isolated tributaries that have high χ relative to neighboring basins. Some of these high- χ tributaries have no side channels, indicating that the lateral divides are moving inward, starving side channels of drainage area, and transforming basin side slopes into unchanneled surfaces (for example, Fig. 5C). These tributaries appear to be victims of the positive feedback associated with area loss, in which loss of drainage area lowers erosion rate and increases χ , leading to surface uplift relative to neighboring basins, and increased vulnerability to further area loss.

There is also evidence for larger-scale basin reorganization. Figure 5B shows a basin that appears to be growing in all directions at the expense of its neighbors. The growing basin has steep upper channels and many landslide scars,

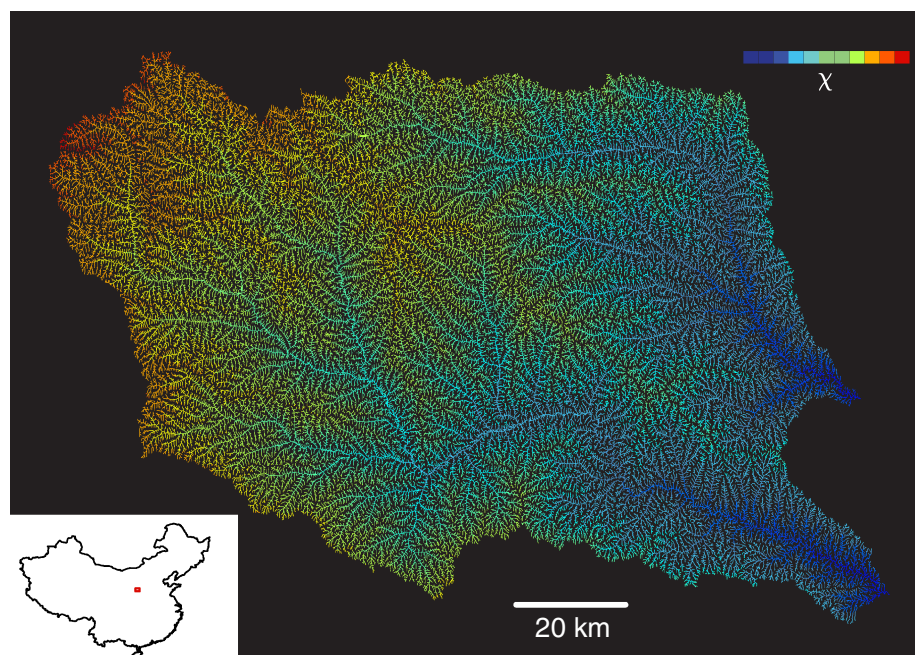


Fig. 4. Map of χ for part of the Loess Plateau, China. Illustrated are the Yanhe River (south) and the Qingjian River (north) basins, two tributaries of the Yellow River. Inset shows the location of the basins within China. See Materials and Methods for details on the calculation of χ . Minimal differences in χ across divides at all scales indicate that basin topology and geometry are near equilibrium.

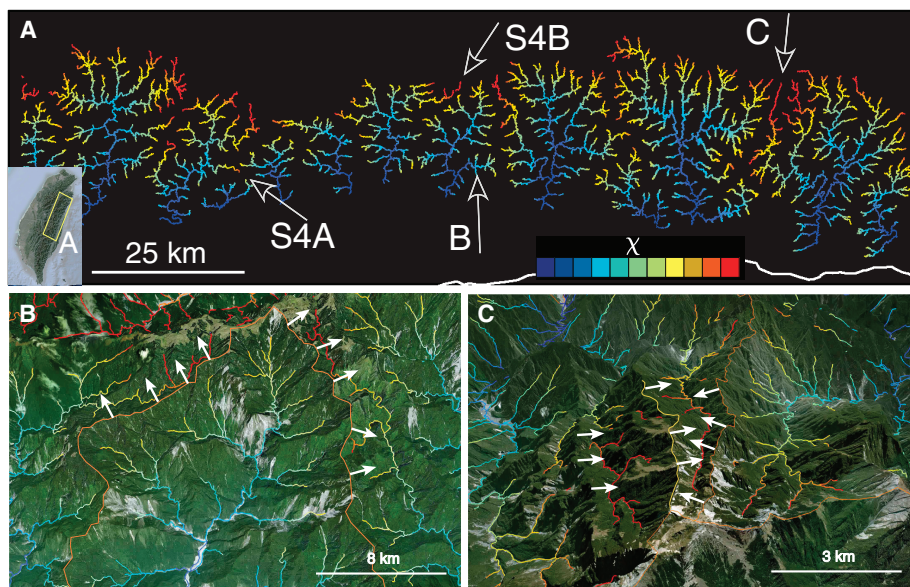


Fig. 5. Map and perspective views of χ for part of the eastern Central Range, Taiwan. (A) Map of χ for a portion of the eastern Central Range (location in inset). Arrows show viewing directions for subsequent panels and for additional figures in supplementary materials. (B) Small basin growing at the expense of neighboring basins. Both neighboring basins with high- χ upper reaches are losing area and may soon lose their headwaters by capture. (C) Long, narrow river basin losing area from several directions, leaving it with high χ and high elevation. Vertical exaggeration in (B) and (C) is 2x. Inferred divide motion from low to high χ is indicated by white arrows.

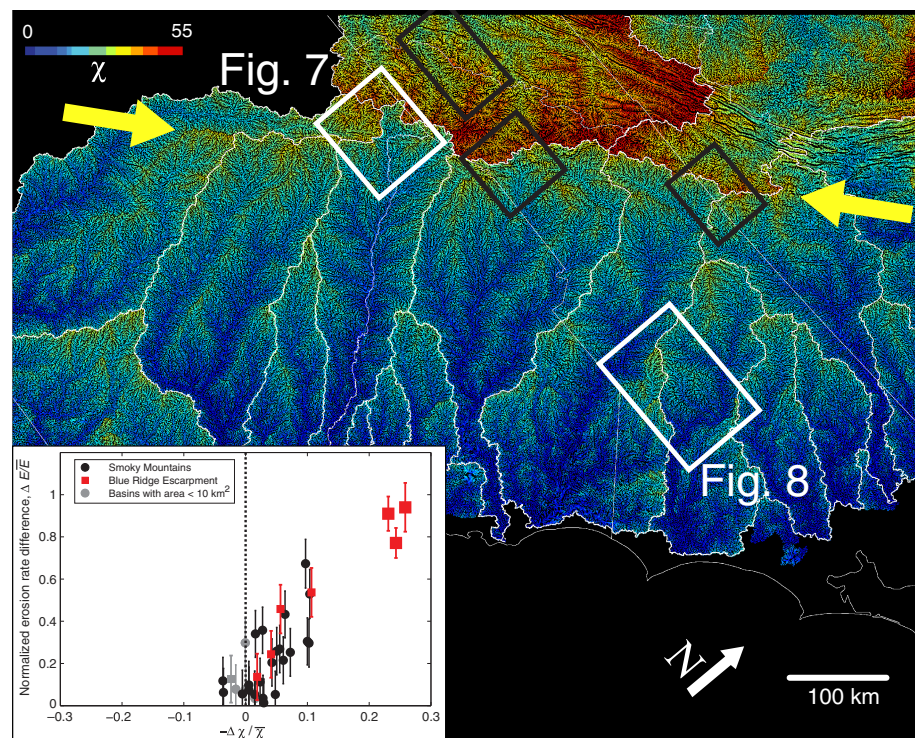


Fig. 6. Map of χ in river basins of the southeastern United States. Heavy white lines show drainage basin boundaries, and fine white lines show state boundaries. The distinct, near-linear discontinuity in χ between the yellow arrows illuminates the Blue Ridge escarpment and its southern continuation into the North Georgia mountains. The χ contrast indicates northwest migration of the escarpment. White boxes mark the areas shown in Figs. 7 and 8. Black boxes mark regions analyzed in the inset figure, which shows differences in basin-averaged erosion rates determined from concentrations of cosmogenic radionuclides (CRNs) in river sediment (10) plotted against differences in χ across the divides separating the basins (see Materials and Methods). Larger red symbols represent averages across the main escarpment. Points to the right of the dashed line are consistent with the inferred direction of divide motion. Data for this figure are shown in supplementary materials.

indicating rapid channel incision. Both neighboring rivers appear to be losing drainage area in their upper reaches based on the existence of perched channels with high χ values. This suggests that even at the largest scale, the major river basins of the eastern Central Range appear to be adjusting their relative sizes, changing shape, and perhaps even increasing their number through expansion of smaller basins.

Comparison of χ and topography across divides also serves to confirm that there are no large changes in K or U through the study area. If this were the case, we would not expect to see the high degree of internal consistency between drainage divide asymmetry and cross-divide χ . Although we do recognize some instances where lithologic effects change channel steepness, these appear to be of minor importance compared to the geometric disequilibrium reflected in χ .

Ongoing Basin Reorganization on a Passive Margin: Southeastern United States

The southeastern seaboard of the United States comprises the coastal plain and Piedmont physiographic provinces and is defined by the old mountain belt of the Appalachians in the west and by the Atlantic Ocean in the east, which formed by rifting starting about 200 million years ago (35). The boundary between the Piedmont and the Appalachians is the Blue Ridge (36, 37), which defines the mountain front from Georgia to Pennsylvania and is interpreted to be an erosional escarpment associated with Atlantic rifting, similar to escarpments on other rifted margins (38). The top of the Blue Ridge defines the water divide between the Atlantic coastal rivers and the Mississippi River basin. This divide appears to be migrating inland, often by discrete capture of upland rivers (15, 23, 36, 39). The spatial distribution of χ illuminates the escarpment beautifully (Fig. 6), with a strong discontinuity in χ between high values in the upper Tennessee River and Ohio River basins and low values in all basins draining directly to the east coast. This discontinuity defines an erosional front propagating to the northwest.

Some care must be taken in interpreting contrasts in χ across the main Appalachian divide, given that rivers that meet at this divide follow very different paths to the ocean and may therefore traverse regions with different uplift, climate, or rock type. If these variations were known precisely, they could be included in the characteristic parameter (Eq. 5). However, even without precise constraints on the spatial patterns of U and K , it is possible to show that the χ difference across the main divide is largely a signature of geometric disequilibrium. The most important heterogeneity that potentially creates spatially variable K is the contrast between the hard, erosion-resistant metamorphic and sedimentary rocks of the Appalachians and the softer sedimentary rocks of the Piedmont and coastal plain (40). If this effect were included through Eq. 5, the result would be to enhance the contrast in χ across the main divide, not diminish it.

Uplift in this tectonically quiescent region is dominated by isostatic response to erosion, which, if not constant, only varies over long wavelengths. However, there may be an additional, relatively young component of dynamic topography (41). This dynamic component may create a spatial gradient in U , but this gradient is oriented predominantly northeast-southwest along the Appalachian crest in the study area (41) and, hence, should not contribute to discontinuities in χ across the main divide. As with the Taiwan example, we can also compare more subtle features of the χ map and topography to check for consistency. For example, the divides between the Apalachicola River, the Tennessee River, and the Altamaha River (Fig. 6) show different values or even signs in cross-divide χ , and in all cases, the contrast is mimicked by subtle but clear asymmetry in topography. These observations suggest that the major features of the χ map do not arise from spatially variable K or U .

We also find support for inferred divide motion in erosion rates derived from cosmogenic nuclide concentrations in river sediment (10). Concentrations of ^{10}Be generated by cosmic ray exposure of quartz, now found as sand in the modern rivers, have been used to estimate erosion rates in a number of catchments across the southern Appalachians. We have compiled published data from the Great Smoky Mountains of Tennessee and North Carolina (42) and two segments of the Blue Ridge escarpment in North Carolina and Virginia (43) (Fig. 6). Motion of a drainage divide must ultimately be driven by different erosion rates on opposite sides of the divide. By comparing erosion rates from adjoining basins, we can estimate the direction of divide motion and test whether it is consistent with the direction inferred from the cross-divide difference in χ (see Materials and Methods). We found that the cross-divide χ difference correctly predicts the difference in erosion rate in 29 of the 34 adjoining basins we studied, with a strong correlation in magnitude in addition to predicting the correct sign (Fig. 6, inset).

Mapping of χ is also useful for identifying discrete capture events, many of which have been recognized along the Appalachian front. We present a documented example in Fig. 7 to show the signature pattern in χ . The Savannah River is proposed to have captured the headwaters of the Apalachicola River. Evidence for this interpretation includes the deeply incised Tallulah Gorge above the capture point and a common freshwater fauna in both catchments (39, 44). The spatial distribution of χ shows sharp discontinuities across the Savannah-Apalachicola and Savannah-Tennessee divides, implying ongoing advance of the Savannah into both of these catchments (Fig. 7). In contrast, the Apalachicola-Tennessee divide is close to equilibrium. A discrete river capture involves a sudden transfer of drainage area from one basin to another, which will strongly affect χ but not elevation, thereby pushing the relationship between χ and elevation in opposite

directions for the captured and capturing rivers (Figs. 1 and 2). This is demonstrated clearly in the Savannah capture region by tributaries to the upper Savannah, which gained area and therefore have smaller χ than expected for their elevation, and by the Apalachicola, which lost area and therefore has larger χ than expected for its elevation (Fig. 7). At present, the upper Savannah is responding to the increase in its drainage area by incising rapidly, creating marked features like the Tallulah Gorge as it lowers its elevation profile back to the regional χ - z trend. Similarly, the Apalachicola is incising less rapidly to raise its elevation profile back to the regional trend. This process will continue until both rivers reach equilibrium or, more likely, another capture occurs.

Another interesting feature of the map in Fig. 6 is the set of chevron-shaped drainage divides

scattered across the coastal plain and Piedmont from Florida to Virginia, which are highlighted by consistently higher χ values on the coastal sides of the divides (Fig. 8). The anomalously high χ values in these basins are associated with anomalously high elevations (Fig. 8), similar to the area-starved basins in Taiwan (Fig. 5), but in this case, the topography has relief of only meters to tens of meters. The coastal basins are losing area as their headwaters narrow and shorten, and some may eventually disappear. This appears to be part of a regional basin reorganization in which the large Atlantic-draining basins are widening and pinching out the smaller, intervening basins. Such a process is consistent with northwest-southeast lengthening of the Piedmont and coastal plain provinces. River basins exhibit a narrow range of length-to-width ratios (45), so that lengthening

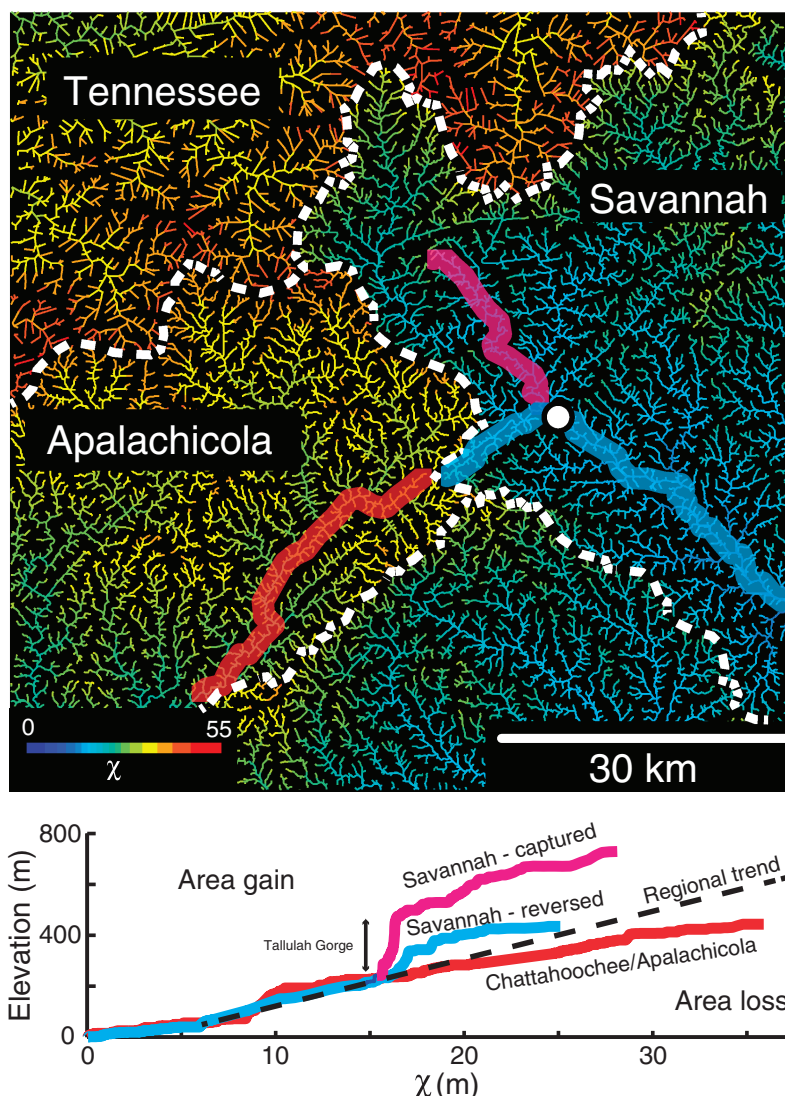


Fig. 7. The Savannah and Apalachicola river capture. The Savannah River has captured the headwaters of the Apalachicola. The white circle marks the capture point. Profiles of elevation against χ (lower panel) are offset from the regional trend in directions consistent with river capture (compare with Fig. 2). The upper reach of the Apalachicola (victim) has anomalously high χ , consistent with area loss, whereas the upper reaches of the Savannah (aggressor) have anomalously low χ , consistent with area gain.

in the flow direction can lead to basin reorganization, decreasing the number of basins to increase the average width (17, 18, 46). The Atlantic coastal region is lengthening by retreat of the Blue Ridge escarpment, as well as by seaward advance of the coastline, as evidenced by uplifted Pliocene terraces, possibly as a dynamic response to deep mantle flow (41).

Global Implications

Our analysis presents and quantifies a dynamic view of landscapes. Drainage divides migrate or make discrete jumps; river basins expand, contract, and deform; and this dynamic reorganization can persist for hundreds of millions of years, or perhaps indefinitely in the presence of active tectonic deformation.

This evidence of shifting drainage divides helps explain well-known morphologic properties of drainage basins. Basins in varied geographic settings are observed to take on specific geometric forms or statistical states (45–47). In particular, characteristics such as length-area scaling, tributary junction angles, and fractal dimension appear to be globally consistent. Models that optimize local or global energy dissipation reproduce many of these fundamental network characteristics (48, 49), although natural basins seem to be slightly suboptimal by these energy metrics. It has long

been suspected that divide migration and river capture might be mechanisms by which drainage basins approach statistically uniform geometry (50). Our numerical models and analyses of natural landscapes provide evidence that this may, in fact, be the case, and additionally suggest that many natural basins are suboptimal because they have not yet reached an equilibrium configuration. Furthermore, our studies suggest that it is the geometric characteristics of the channel networks and their bounding divides that drive the system toward equilibrium.

The ability to map disequilibrium in river basins also has implications for other fields of study. For example, river profiles are often interpreted in terms of transient changes in tectonic uplift, climatic conditions, or sea level. However, our analysis suggests that many perturbations in river profiles may instead arise from changes in drainage area. Drainage network connections and water divides that form geographic barriers affect the transport of sediment, nutrients, and dissolved elements, including those with important global biogeochemical functions such as nitrogen and carbon. Similarly, the migration or diversification of aquatic species and entire ecosystems depends on the transport pathways defined by rivers. Our ability to identify past and ongoing changes in river networks creates a new opportunity to ex-

plore connections between geological, chemical, and biological systems.

Materials and Methods

Response of χ to a Change in Drainage Area

For the calculation in Fig. 2, we assume that area and channel length scale according to $A = k_a x^h$ with $x = 0$ at the water divide (51), and to keep the calculation analytical, we assume that $h = 2$ and $m/n = 0.5$. Most other values for the coefficients h , m , and n would require numerical integration, but the results will not change in character for a reasonable range of these coefficients. Defining a nondimensional length $x^* = \frac{x}{x_d}$, we obtain an initial distribution of

$$\chi_{\text{init}} = \sqrt{\left(\frac{A_0}{k_a}\right)} \int_{x^*}^1 x^{-1} dx = -\sqrt{\left(\frac{A_0}{k_a}\right)} \ln(x^*) \quad (6)$$

and a steady-state elevation linearly proportional to χ_{init} . We consider an instantaneous change in area of ΔA of this basin, assumed to be the consequence of adding or removing area to the headwaters of the basin. The change in area can be positive or negative, but it must occur upstream of the analysis. The width of the basin downstream of the area change is unchanged. With the nondimensional area perturbation, $A^* = \frac{\Delta A}{k_a \chi_d^2}$, following the area perturbation is defined by

$$\chi_p = \sqrt{A_0} \int_{x^*}^1 \frac{1}{\sqrt{k_a x^2 + A^*}} dx \quad (7)$$

which can be integrated to give

$$\chi_p = -\sqrt{\frac{A_0}{k_a}} \ln \left(\frac{x^* + \sqrt{(x^*)^2 + A^*}}{1 + \sqrt{1 + A^*}} \right) \quad (8)$$

Plotting χ_{init} against χ_p (Fig. 2) is equivalent to showing elevation against χ for an instantaneous change in area. For a basin losing area, there is a corresponding decrease in length of the channel, hence the smaller extent in χ_p of the curves in Fig. 2. For the basin gaining area, there is a corresponding increase in channel length, but this is not shown in Fig. 2 because the actual slope and length of this segment depend on the area distribution of the captured channel. A plot of channel slope against drainage area shows a similar perturbation (fig. S1).

Landscape Evolution Model

The numerical model for landscape evolution (Fig. 3) uses the code DAC described by Goren *et al.* (21). DAC incorporates numerical and analytical solutions to represent processes at different scales. The numerical component solves the conservation of mass equation with a stream power incision law (Eq. 1) for a channel network spanning a dynamic, irregular grid. An analytical solution is used for the subgrid topography to represent low-order fluvial

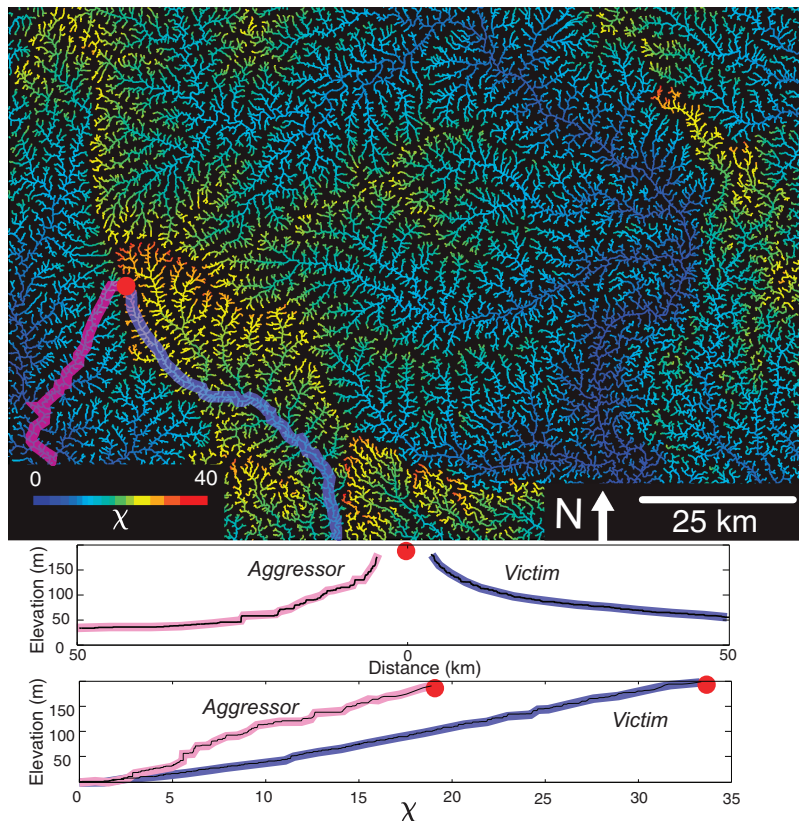


Fig. 8. Disequilibrium basins of the North Carolina coastal plain. The basin at the center of the map has a chevron-shaped drainage divide with high χ values on the interior, coastal side. Lower panels show elevation and χ profiles for two rivers that meet at the divide. The river to the east (victim), interior to the chevron, has systematically higher elevation and χ , indicating progressive loss of drainage area and slower erosion rate (compare with Fig. 1). A similar feature is visible in the upper right of the map.

channels and hillslopes. The channel network and the numerical grid evolve as divide migration leads to river capture, abandonment of channels with insufficient drainage area, or creation of new channels as hillslopes lengthen. The model in Fig. 3 consists of a 50×100 -km rectangular domain in which the four edges are fixed to a constant elevation. Precipitation and rock type are steady and uniform. An initial topography is generated by imposing a rock uplift rate that varies linearly from 0.5 mm/year along the lower edge to 5.0 mm/year along the upper edge. These conditions are run to steady state. Subsequently, the tectonic gradient is removed, and uplift rate is set to a constant value of 1 mm/year. Basin divides on all scales become unstable and migrate, causing drainage basins to change their size and shape (see movie S1). χ is calculated from Eq. 3 on the discrete grid, but because part of the river channel network is contained in the analytical solution, sections of the uppermost catchments are not shown in Fig. 3 or movie S1.

Construction of χ Maps

Construction of maps of χ followed a specific protocol. Hydraulic attributes of base level, flow directions, flow paths, and accumulated flow (upstream drainage area) were extracted from a digital elevation model (DEM). For flow direction and paths, closed basins were filled, and steepest descent neighbors were found for local flow direction. Any pixel with a contributing area less than a critical value, A_c , was excluded from the analysis. The critical area was typically on the order of 10^6 m², although it can vary depending on the DEM resolution and the drainage density of the landscape. This value does not affect the downstream value of χ , but it does determine how high into a catchment we conduct the analysis. An arbitrary scaling area, A_0 , and a value for m/n were selected, followed by integration of Eq. 3 to determine χ for all pixels in the domain.

The concavity, m/n , was selected through an iterative process. First, we constructed a series of χ plots for individual drainage basins, covering a range of concavity values, and noted the m/n value that best reduced the scatter (29, 52). This was used to construct an initial χ map, which was then interpreted in terms of divide stability. If divides appeared stable within the basin of interest, the process was complete. However, if there were large contrasts in cross-divide χ , we reconsidered the χ plots, noting the profiles of channels that appeared to be gaining or losing area based on the χ analysis, or on independent geologic, geomorphic, or geochemical data. Because these χ plots are expected to curve up or down (Figs. 1 and 2), we modified our selection of m/n accordingly. The interpretation of area gain or loss is partially based on the resultant χ map, so is potentially circular, but the iterative process consistently converged to a single solution. In some cases, we also applied this method to individual channel profiles where we could make an a priori assumption regarding the shape of the χ plot. As a final check, we overlaid the χ map on the digital

topography and checked that predicted cross-divide χ discontinuities were consistent with topographic features such as asymmetric upper channel profiles across divides. Figures 5 and 8 are examples of such comparison.

Loess Plateau

The 90-m resolution DEM derived from the Shuttle Radar Topography Mission (SRTM) (53) was used for the Loess Plateau. Two drainage basins were extracted and analyzed using a critical area, A_c , of 0.05 km² and a scaling area, A_0 , of 1 m². The low value for the critical area reflects the high drainage density of the area. Interpretation of the resultant χ -elevation plots gave an optimum m/n of 0.35 (fig. S2).

Taiwan

For Taiwan, we used a 40-m resolution DEM derived from aerial photographs and available from the Center for Space and Remote Sensing Research, National Central University, Taiwan. A scaling area, A_0 , of 1 m² and a critical area, A_c , of 1 km² were used. Plots of χ -elevation were constructed with concavity varying from 0.0 to 0.6. The smallest scatter is obtained for a concavity of 0.3 to 0.4, but we favor a slightly higher value. Given our finding of many moving divides and captures, we expect many channels to exhibit χ plots with low slope and high χ , as well as many channels with kinks in the χ -elevation profile in response to capture. These features become more obvious with concavities of 0.45 to 0.55 (fig. S3), so we prefer this value and use a value of 0.5 for the maps of χ . This value was confirmed by comparison with topography and imagery, which is quite marked in Taiwan (Fig. 3 and fig. S4).

Southeastern United States

For the analysis of the southeastern United States, we used the 90-m CGIAR SRTM DEM, analyzed with a critical area, A_c , of 0.3 km² and a scaling area, A_0 , of 1 m². We constructed χ -elevation plots for individual drainage basins (identified in fig. S5) in the study area for a range of m/n values (figs. S6 to S9). We found very dynamic divides in the region with examples of basins that were dominantly growing and others that were dominantly shrinking. The minimum variance in the χ -elevation plots was found for values of m/n from 0.25 to 0.35, but to match the χ -elevation plot concavity to our interpretation of growing and shrinking basins, we required a larger m/n value of 0.4 to 0.5. We also conducted a similar analysis on individual rivers where geological or geomorphic evidence permitted an a priori assessment of capture or a moving divide. This exercise also gave m/n values of 0.4 to 0.5 (for example, fig. S10). Maps in this paper were constructed using an m/n of 0.45, though other values were constructed to test sensitivity to m/n (fig. S11), which revealed that the features described in this paper were robust over a large range of m/n values.

Map construction on the western side of the Appalachians is complicated by the need to in-

clude the drainage area of the entire Mississippi River basin. To avoid integrating the alluvial lower Mississippi, we initiated the χ integration at the confluences between the westward draining rivers and the Ohio River. This required selecting a χ -elevation pair for each river. To calculate these values, we used the regional χ -elevation trend for the lower Tennessee River and lower Kanawha River. This resulted in an initial point of 8 m in χ and 100 m in elevation for the Tennessee River and 10 m in χ and 148 m in elevation for the Kanawha River.

Differential Erosion Rates Estimated from Cosmogenic Radionuclide Concentrations

We used published concentrations of the CRN ¹⁰Be in quartz river sand to estimate differential erosion rates in select river basins. We used published data from Portenga and Bierman (10) in three areas within the southern Appalachians: two locations along the Blue Ridge escarpment, and one in the Great Smoky Mountains (Fig. 6). Original studies are in (42, 43). We selected locations with CRN measurements in adjoining basins where the difference in erosion rate can be compared to the cross-divide contrast in χ . To estimate differential erosion across a divide, we differenced the basin-averaged erosion rates of adjoining basins and assigned the resulting differential erosion rate, ΔE , to the divide segment common to both basins. We then calculated the mean $\Delta\chi$ across the shared divide segment by differencing pairs of adjoining channel heads in the same direction. This procedure was carried out for all primary divide segments between basins with comparable size. We did not analyze nested sub-basins. We also excluded one basin from the Great Smoky Mountains location, which appeared to have anomalous ¹⁰Be concentrations associated with an unusual intrusive igneous unit (figs. S12 to S14 and table S1).

In the two Blue Ridge locations, there were few basins with CRN data directly adjoining one another. In these locations, we calculated an average differential erosion rate across the escarpment itself. We divided the escarpment into three segments (two segments as shown in fig. S14 and one segment shown in fig. S13) that had erosion rate measurements on each side. We then determined the mean erosion rate of each side of the escarpment by taking the mean erosion rate of basins proximal to the escarpment, but draining in opposing directions. We differenced these mean erosion rates to calculate an escarpment-averaged ΔE . We then calculated the mean $\Delta\chi$ across each escarpment segment by differencing, in the same direction, the mean channel head χ values along the escarpment segment. The results from all three study areas were normalized by dividing ΔE and $\Delta\chi$ by their respective mean E or χ for the two basins being compared (Fig. 6, inset).

We also tested how much of the overall variance in the CRN erosion rate data could be explained by the cross-divide χ gradients. We calculated an average cross-divide $\Delta\chi$ for a basin by

integrating the difference of channel head χ across the exterior perimeter of each basin. This provides an aggressivity metric, in which positive values indicate basins that are likely growing at the expense of their neighbors, whereas basins with negative values are victims that are likely to be shrinking. These aggressivity indices are compared with basin-wide erosion rates in fig. S15.

Movie S1. Numerical simulation showing the evolution of χ during landscape reorganization.

Data Archive. Chi maps for each region can be downloaded in kml format from www.sciencemag.org/content/343/6175/1248765/suppl/DC1. Data details are given in table S2.

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Supplementary Materials

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Innate Immune Activity Conditions the Effect of Regulatory Variants upon Monocyte Gene Expression

Benjamin P. Fairfax *et al.*

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Innate Immune Activity Conditions the Effect of Regulatory Variants upon Monocyte Gene Expression

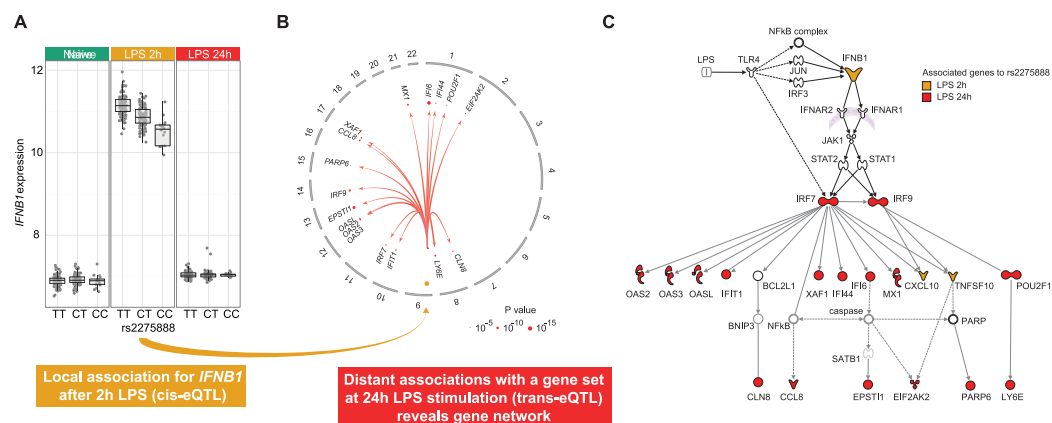
Benjamin P. Fairfax,* Peter Humburg, Seiko Makino, Vivek Naranbhai, Daniel Wong, Evelyn Lau, Luke Jostins, Katharine Plant, Robert Andrews, Chris McGee, Julian C. Knight*

Introduction: Many genetic variants associated with common disease susceptibility occur close to immune-related genes in noncoding DNA, suggestive of a regulatory function. The definition of functional variants and the specific genes that they regulate remains challenging and in many cases is unresolved. We hypothesized that a significant proportion of variants, including those implicated in disease, may show activity in a context-specific manner and therefore only be identifiable upon triggering of immune responses.

Methods: We mapped interindividual variation in gene expression as a quantitative trait, defining expression quantitative trait loci (eQTLs). To investigate the effect of innate immune stimuli on eQTLs, we exposed primary CD14⁺ human monocytes from 432 European volunteers to the inflammatory proxies interferon- γ (IFN- γ) or differing durations (2 or 24 hours) of lipopolysaccharide (LPS). eQTL mapping was performed on a genome-wide basis with an additive linear model. A subset of 228 individuals with expression data available for all experimental conditions enabled cross-treatment comparisons.

Results: Stimulation with LPS or IFN- γ resulted in profound effects across monocyte eQTLs, with hundreds of genes and associated pathways demonstrating context-specific eQTLs dependent on the type and duration of stimulus. Context-specific eQTLs frequently intersected established canonical pathways of monocyte signaling and included key nodal genes and effector molecules. These eQTLs are typically more distal to the transcriptional start site and, in some cases, showed reversal of effect between conditions. We also found stimulation reveals novel eQTLs with simultaneous effects involving many genes (trans-eQTLs). Examples included coding polymorphisms in *CYP1B1*, *P2RY11*, and *IDO2* that modulate activity and develop trans network effects upon stimulation; an LPS-specific IFN- β cytokine network response driven by a cis-eQTL for *IFNB1* that was only revealed over time; an interferon regulatory factor 2 (IRF2) transcription factor modulated network up-regulated by IFN- γ involving a cis-eQTL for *IRF2*; and an IFN- γ -inducible trans gene network involving the transcription factor NFE2L3. We find trans associations to the major histocompatibility complex are dependent on context, paralleling the expression of class II genes. Induced eQTLs were enriched for disease-risk loci with context-specific associations to many putative causal genes including at *ATM*, *IRF8*, and *CCR3*. Conditional analysis defined additional independent stimulus-specific peaks of association for a given gene. For *CARD9* we observed, in addition to a constitutive eQTL informative for a genome-wide association study locus for Crohn's disease, a stimulus-specific peak eQTL after IFN- γ , defining a further independent signal of disease association.

Discussion: Interindividual variation in immune responses is accompanied by diverging patterns of gene regulation dependent on underlying genotype. In human monocytes, many regulatory variants display functionality only after pathophysiologically relevant immune stimuli. By considering the cellular and environmental context relevant to disease, it is possible to more extensively resolve functional genetic variants and the specific modulated genes associated with disease.



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FIGURES IN THE FULL ARTICLE

Fig. 1. Genotype modulates the gene expression response to innate immune stimuli in monocytes.

Fig. 2. Trans-eQTL demonstrate context specificity and identify master regulatory loci after treatment.

Fig. 3. Temporal effects for a stimulus-specific trans-eQTL.

Fig. 4. Cis regulation of *IRF2* at rs13149699 has profound transcriptional consequences in trans.

Fig. 5. Stimulus-specific eQTL and GWAS.

Fig. 6. Examples of context-specific eQTL informative for disease risk.

SUPPLEMENTARY MATERIALS

Materials and Methods

Figs. S1 to S12

Tables S1 to S7

References

Context-specific genetic association with differential gene expression in IFN- β signaling. (A) A local association (cis-eQTL) with *IFNB1* expression for a single-nucleotide polymorphism (rs2275888) revealed after 2 hours of LPS stimulation of monocytes. (B) This genetic marker shows association with expression of 17 genes on different chromosomes (trans-eQTLs) after 24 hours of LPS stimulation, forming a gene network (C) consistent with the IFN- β signaling cascade.

The list of author affiliations is available in the full article online.

*Corresponding author. E-mail: bfairfax@well.ox.ac.uk (B.P.F.) and julian@well.ox.ac.uk (J.C.K.)

Innate Immune Activity Conditions the Effect of Regulatory Variants upon Monocyte Gene Expression

Benjamin P. Fairfax,^{1*} Peter Humburg,^{1†} Seiko Makino,^{1†} Vivek Naranbhai,¹ Daniel Wong,¹ Evelyn Lau,¹ Luke Jostins,¹ Katharine Plant,¹ Robert Andrews,² Chris McGee,² Julian C. Knight^{1*}

To systematically investigate the impact of immune stimulation upon regulatory variant activity, we exposed primary monocytes from 432 healthy Europeans to interferon- γ (IFN- γ) or differing durations of lipopolysaccharide and mapped expression quantitative trait loci (eQTLs). More than half of cis-eQTLs identified, involving hundreds of genes and associated pathways, are detected specifically in stimulated monocytes. Induced innate immune activity reveals multiple master regulatory trans-eQTLs including the major histocompatibility complex (MHC), coding variants altering enzyme and receptor function, an IFN- β cytokine network showing temporal specificity, and an interferon regulatory factor 2 (IRF2) transcription factor–modulated network. Induced eQTL are significantly enriched for genome-wide association study loci, identifying context-specific associations to putative causal genes including *CARD9*, *ATM*, and *IRF8*. Thus, applying pathophysiologically relevant immune stimuli assists resolution of functional genetic variants.

Inappropriate immune activity and associated inflammation are involved in the pathogenesis of a broad range of common diseases including inflammatory bowel disease, atherosclerosis, rheumatoid arthritis, and cancer. Moreover, a significant proportion of common disease risk loci identified with genome-wide association studies (GWAS) implicate immune genes (1). Most GWAS loci consist of single-nucleotide polymorphisms (SNPs) within noncoding, putatively regulatory DNA, often at a distance from any gene coding regions (2). The identification of functional regulatory variants and associated modulated genes is key to interpreting GWAS findings and establishing how genes are associated with disease. This can be explored by mapping gene expression as a quantitative trait (eQTL mapping) (3–6).

The effect of a regulatory variant on gene expression is frequently dependent on the cell and tissue type (7–9). The extent to which specific cellular pathway activation by environmental agents can modulate regulatory variant effects is relatively unexplored. Monocytes are cardinal innate immune cells that constitutively express receptors responsive to pathogens and cytokines including Toll-like receptors (TLRs) (10), with activity being intricately regulated by the cellular environment and interactions with other immune cell types. Upon stimulation, mono-

cytes exhibit large-scale gene transcription with concomitant cytokine production, enhanced antigen processing, and migration (11, 12). Context-specific genetic variants that directly modulate innate immune responses or gain regulatory capacity upon pathway activation have been subject to selective pressure and may affect disease susceptibility (13). It is probable that such variants are widespread, although they are functionally unidentifiable in resting cells. Supporting this, macrophages derived from different mouse strains demonstrate multiple de novo trans-regulatory loci upon inflammatory stimuli (14). We hypothesized that the transformation in monocyte transcriptional activity accompanying innate immune responses would allow us to detect the effect of context-specific regulatory genetic variation upon gene expression in humans.

Results

Innate Immune Stimuli–Induced Transcriptomes

Interindividual variation in the early (2 hours) and late (24 hours) inflammatory responses to lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls that triggers TLR4 signaling (10), and interferon- γ (IFN- γ) (24 hours), a cytokine acting through the JAK-STAT (Janus kinase–signal transducer and activator of transcription) signaling pathway, important in mycobacterial and viral infections (15, 16), was characterized in highly purified primary CD14⁺ human monocytes from 432 individuals of European ancestry. Genome-wide gene expression profiling and genotyping were performed with HumanHT-12 v4 BeadChip (Illumina) and HumanOmniExpress-12v1.0 BeadChip (Illumina).

We tested 609,704 SNPs [minor allele frequency (MAF) > 0.04] and expression data for 15,421 probes for 414 individuals in the naïve state, 367 individuals after exposure to IFN- γ , 322 individuals after 2-hour LPS, and 261 individuals after 2-hour LPS. To allow comparison with naïve monocytes from the same individuals, expression data from untreated, incubated samples from a subset of 59 individuals were used to regress out expression changes attributable to incubation from the treated samples. Data across all four conditions were available from 228 individuals, permitting cross-treatment comparison. As anticipated, treatment induced significant changes in monocyte expression profiles (table S1) with hierarchical cluster analysis demonstrating that group membership was defined by treatment (fig. S1). Analysis of differential gene expression was consistent with biological expectations in terms of identified canonical pathways, the most significant upstream regulators, and the transcriptomic differences between early and late responses to LPS (table S1 and fig. S1) (17).

Identifying Context-Specific Local Genetic Regulatory Variants

Local, likely cis-acting eQTL (referred to as cis-eQTL) were defined as SNPs showing association with gene expression that were located within a 1-Mb window of the associated probe. We tested for association with gene expression using an additive linear model (18). We included surrogate variables consisting of major principal components (PC) of the expression data as covariates to limit the effect of confounding factors. Similar to others (19, 20), we find this enhanced detection of cis- and trans-eQTLs (fig. S2). Using this approach, we found that the majority of all cis-eQTLs observed affected gene expression only upon cell stimulation, and a significant proportion of constitutive eQTL present in naïve cells were no longer present after treatment (Fig. 1, A and B, and table S2). Across all four data sets, most genes show evidence of eQTL, with 83.7% of probes tested (12,905/15,421 probes) showing association under at least one condition [false discovery rate (FDR) < 0.05].

For the 228 individuals for whom we had expression data for all four conditions, we observed 21,516 independent cis SNP-probe associations across data sets (defined as the most significantly associated SNP with expression for a given probe per data set), 24.6% of which had the lowest *P* value for association in naïve cells, 21.6% after 2-hour LPS, 25.4% after 24-hour LPS, and 28.3% after IFN- γ . A total of 11,476 probes had at least one eQTL, of which 43.4% (4977 of 11,476) only had eQTL after monocyte stimulation. We find that eQTL are similarly specific to the unstimulated state, with 54.1% of naïve eQTL (2866 of 5299) not found after treatment (Fig. 1A, fig. S3, and table S2). One explanation for many context-specific eQTL is that treatment increases expression to quantifiable

¹Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, UK. ²Wellcome Trust Sanger Institute, University of Cambridge, Hinxton CB10 1SA, UK.

*Corresponding author. E-mail: bfairfax@well.ox.ac.uk (B.P.F.); julian@well.ox.ac.uk (J.C.K.)

†These authors contributed equally to this work.

levels, thus allowing eQTL detection. To investigate the extent to which this contributed to the observed stimulus specificity of eQTL, we restricted our analysis to probes with detectable expression (Illumina detection score $P < 0.01$) in more than 90% of samples from each data set. We found that 33.4% (1702 of 5082) of these probes only had an eQTL after treatment, indicating that context-specific eQTL are identified because of both treatment-induced regulatory effects and treatment-inducing gene expression to detectable levels. We observed that the range of effect sizes (all eQTL, 6.0 to 94.7%; median, 11.3%) was markedly similar across the different conditions, although IFN- γ -associated

eQTL had slightly larger effect sizes than all others (fig. S4). Cell-specific eQTL more often involve enhancer elements distal to the transcription start site (TSS) when compared to eQTL shared across cell types (7). It is unclear whether eQTL induced by innate immune stimuli show a similar distribution. By analyzing all expression-associated SNPs (eSNPs), we found that the more conditions an eSNP was observed across, the more proximal it was to the TSS ($P < 2.2 \times 10^{-16}$). Even when assessing only significant eSNPs with association below a $P < 5 \times 10^{-8}$ threshold, a similar effect was apparent ($P < 2.9 \times 10^{-11}$). We find that the effect size for an eSNP increases with

proximity to the TSS (fig. S5) and that eSNPs that could only be observed after treatment were significantly more distal than those observed in the naïve state ($P < 2.2 \times 10^{-16}$) (fig. S5), consistent with the pattern previously observed with cell type-specific eQTLs. For a minority of eQTL, associations were observed across conditions, but with the direction of effect switching between those conditions. We mapped the most significant association per condition (17) and identified 20 genes, including *HIP1* and *STEAP4*, with shared peak eQTLs mapping to a single locus but with opposing directions across conditions (Fig. 1C, fig. S6, and table S2).

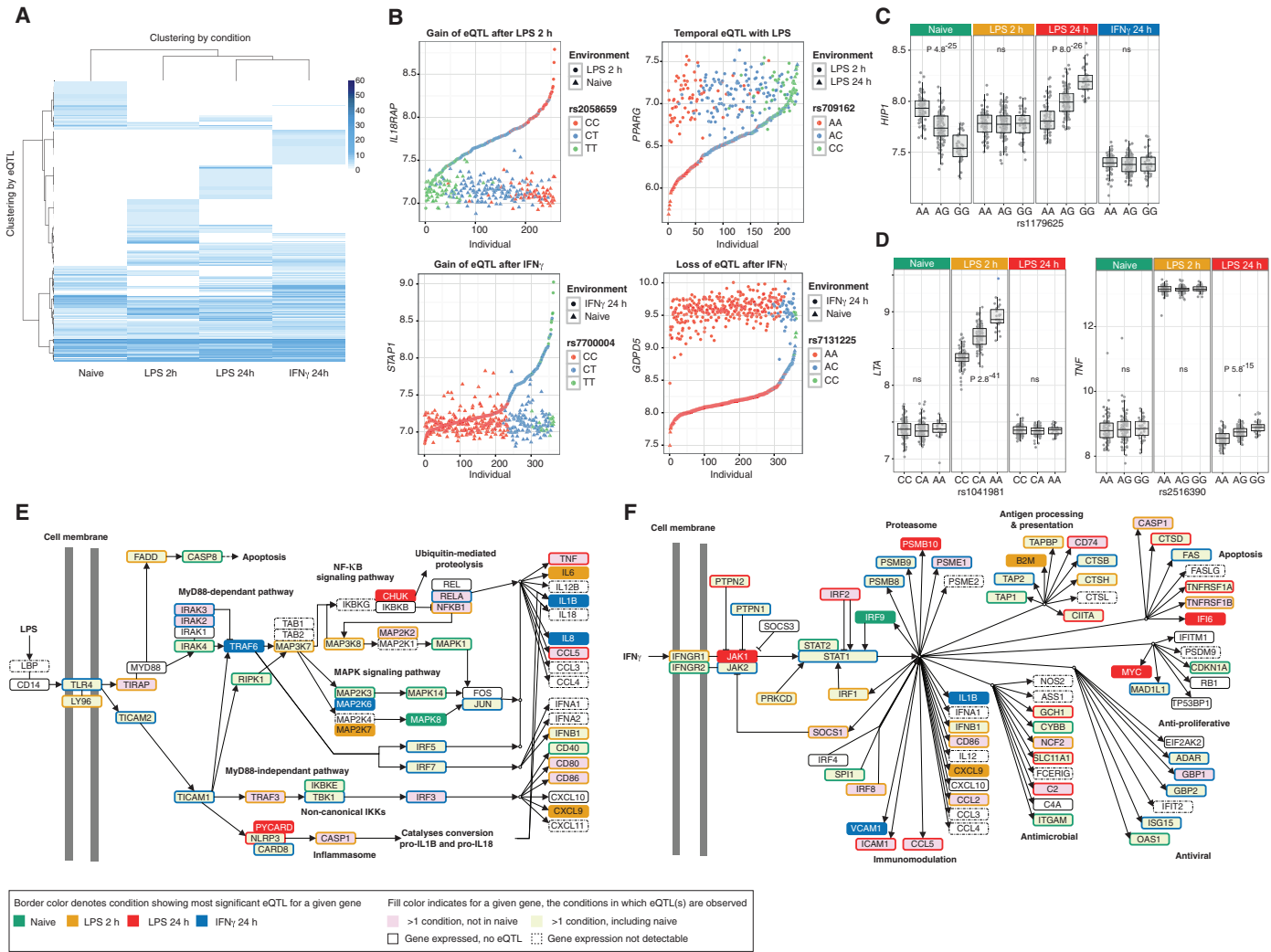


Fig. 1. Genotype modulates the gene expression response to innate immune stimuli in monocytes. (A) Cis-eQTL mapping of 228 individuals with matched expression data available across all four treatments shows that the majority of eQTL are context-dependent. Genes with significant eQTL (FDR < 0.05) are hierarchically clustered along the y axis according to the t statistic of the most significant eQTL present across treatments (along the x axis). (B) Gene expression values before and after treatment plotted pairwise by individual. In clockwise order: 2-hour LPS induces differential expression of *IL18RAP* dependent on rs2058659 genotype; 2-hour LPS treatment results in uniform *PPARG* up-regulation, whereas continued LPS exposure over 24 hours is associated with reduction in expression in

carriers of the A allele of rs709162; an eQTL tagged by rs7131225 found in untreated cells (naïve state) for *GDPD5* transcription could not be detected after gene induction with IFN- γ ; and IFN- γ stimulation leads to induction of *STAP1* expression in carriers of the T allele of rs7700004, relative to the C allele. (C) Cis-eQTL displays context-specific directional effects. rs1179625 is the SNP with the greatest effect on expression of *HIP1* in the naïve state and after 24-hour LPS but exhibits opposing direction of effect. ns, not significant. (D) Induction by LPS reveals stimulus-specific cis-eQTL for *LTA* and *TNF*. (E and F) Genes within the (E) TLR4 signaling and (F) IFN- γ signaling pathways highlighted for those with eQTL for shared data set illustrating context specificity.

A diverse set of immune-related genes were found to show eQTL after induction with LPS or IFN- γ , ranging from transcription factor genes such as *ETS2*, *JUN*, and *IRF2* (*interferon regulatory factor 2*) to key cytokines and receptors such as *IL8* (*interleukin-8*), *IL32*, *IL18R1*, and *FAS* (table S2). For *LTA*, an eQTL was observed after 2-hour LPS, whereas no eQTL was apparent for *TNF* on induction at 2 hours but was seen on down-regulation after 24-hour LPS (Fig. 1D). We investigated the relationship of specific treatments with observed eQTLs in terms of canonical gene pathways. This resolved contiguous pathways of induced eQTL activity (fig. S7). For the TLR4 pathway, eQTL specific to treatment were revealed for nodal genes including *TIRAP*, *TRAF6*, *FADD*, and MAP (mitogen-activated protein) kinase genes, whereas genes encoding key components of the inflammasome such as *CASP1* and *PYCARD*, as well as downstream cytokines including *IL6*, *CXCL9*, and *IFNB1*, showed eQTL specific to early and late responses to LPS (Fig. 1E). Similarly, treatment revealed eQTL in nodal IFN- γ signaling pathway genes and many downstream genes involved in the proteasome, apoptosis, antigen processing, and presentation (Fig. 1F).

Trans-eQTL and Stimulus-Specific Hotspots

Associations involving distant genes (trans-eQTL, defined as SNPs >1 Mb from the probe) may define gene networks regulated by specific SNPs and can provide unbiased insights into pathway identity and underlying biological processes. It is unclear whether the relative paucity of trans-eQTL observed in human eQTL studies, usually attributable to limited power, may also reflect that many trans networks are undetectable in baseline states (21). Our genome-wide eQTL mapping across stimuli in the full data sets, correcting for multiple testing, resolved a total of 1838 trans-associating genes at FDR <0.05 (394 genes at $P < 5 \times 10^{-12}$, approximate to Bonferroni-corrected $P = 0.05$). Trans-associating loci showed a high degree of context specificity—most notably after IFN- γ treatment when multiple novel trans-eQTLs were identified that were frequently unobservable in the naïve state (Fig. 2, fig. S8, and table S3). Several loci, such as the previously described 12q15 locus at *LYZ* (8, 22) and the major histocompatibility complex (MHC), showed significant trans associations across treatments, whereas other master regulatory SNPs were resolved only in LPS or IFN γ -induced cells and putatively driven by cis-eQTL modulating cytokine release, enzymes, and transcription factors (Fig. 2).

Stimulus Specificity for MHC Trans-eQTL

The MHC has been previously associated with trans-eQTL in primary tissues (8, 19). Trans associations to the MHC predominantly map to SNPs in the class II region and demonstrate plasticity across stimuli (Fig. 2 and fig. S9), with 45 genes mapping in trans (FDR < 0.05)

to the class II region after IFN- γ treatment in the paired 228 data set, whereas after 24 hours of exposure to LPS, only one observation is made. Moreover, after IFN- γ treatment, we see the same trans-associating genes as in the naïve state, but almost all show increased effect sizes. The number of trans-associated genes mirrors class II expression, where IFN- γ and chronic LPS are associated with robust increases and suppression of class II gene expression, respectively (23) (fig. S9), suggesting that relative expression of class II genes may control trans effects.

Temporal Resolution of Cis- and Trans-eQTL Involving IFN β Signaling

Cis-eQTL regulating cytokine release could be envisaged to affect expression of cytokine-modulated genes in the inflammatory cascade resulting in dose response-invoked eQTL in trans over time. An example of such temporal effects involves *IFNB1*, encoding the cytokine IFN- β , which exhibits a temporal relationship between genetic modulators and gene networks (Fig. 3). After 2-hour LPS (Fig. 3A), we observe a local cis-eQTL for *IFNB1* at rs2275888; the same SNP associated in trans after 24-hour LPS with *IRF9*, *IFI6*, *EPSTI1*, *LY6E*, and *MX1* expression.

We further interrogated trans associations to rs2275888 on a single SNP basis. This revealed that most associations occur after 24-hour LPS, with 17 genes in trans, many of which were archetypal early interferon response genes (Fig. 3, B and C, and table S4). Ingenuity pathway analysis (IPA) of trans-associated genes after LPS stimulation resolved an IFN- β -driven network and identified a key role for the upstream regulator IRF7 ($P = 9.5 \times 10^{-26}$) (Fig. 3D). Antiviral response was the most significant disease and biological annotation ($P = 1 \times 10^{-16}$), whereas significant canonical pathways included interferon signaling ($P = 5.8 \times 10^{-8}$) and pattern recognition receptors (recognition of bacteria and viruses) ($P = 6.7 \times 10^{-08}$). This temporal relationship between a cis-eQTL and trans-associated gene network is consistent with the downstream consequences of differential IFN- β expression after LPS induction modulated by rs2275888.

Coding Polymorphisms Associate with Stimulus-Specific Trans Networks

We identified stimulus-specific trans networks putatively driven by coding variants. One such example is a trans hub observed at rs1800440, which codes for an amino acid substitution (p.Asn453Ser) in the first exon of *CYP1B1*, encoding a monocyte-expressed cytochrome P450 enzyme (24). This polymorphism is associated with a reduction in the half-life of CYP1B1 to one-third that of wild type (25) and is a cis-eQTL for *CYP1B1* (table S2), consistent with reduced half-life driving a compensatory increase in transcription of the variant allele. Single SNP analysis of rs1800440 identified

43 genes, predominantly implicated in cancer and the inflammatory response, associating in trans after IFN- γ treatment (table S4). This variant shows significant variation in interpopulation allele frequency, the minor allele being very rare in African and Asian populations [fixation index (FST) of 0.288 ($P = 0.03$) and integrated haplotype score (iHS) of -2.126 for the CEU (Caucasian) HapMap population], suggesting possible selection pressure.

Similar examples of structural variants driving trans networks were identified after IFN- γ up-regulation of *IDO2* involving a p.Arg248Trp substitution in exon 9 (rs10109853), which results in a 90% reduction in tryptophan catabolic activity (26), and after LPS induction to a p.Ala87Thr variant (rs3745601) in *P2RY11* modulating ligand binding (27) (fig. S8 and table S3).

Gene Targets of the Transcription Factor IRF2 Modulated by a Stimulus-Specific Cis-eQTL

Cis-eQTL that regulate transcription factor abundance may generate associations in trans to transcription factor target genes (8, 19, 22, 28, 29). We report a significant context-specific trans gene hub to chromosome 4q35 tagged by rs13149699. This SNP displayed a strong stimulus-specific cis association with *IRF2*, encoding the transcription factor IRF2. Carriage of the A allele of rs13149699 resulted in LPS-induced up-regulation of *IRF2* expression, whereas the G allele was associated with coincident suppression of expression. IFN- γ stimulation caused up-regulation of both *IRF2* alleles, although more markedly so in A allele carriers ($P = 6.9 \times 10^{-32}$) (Fig. 4A). The ENCODE data set (30) shows that rs13149699, which is located in an intergenic region 57 kb telomeric to *IRF2* (Fig. 4B), is spanned by a deoxyribonuclease I (DNase I) hypersensitive site in primary CD14⁺ monocytes and binds a cluster of transcription factors including REL-A (nuclear factor κ B p65) (fig. S10). In the stimulated cells, this SNP is associated with a major trans network, and on single SNP analysis, more than 300 trans genes were defined in the complete data sets (Fig. 4, C to E, and table S4). Analysis of paired samples showed no probes associating in the naïve cells, implicating treatment-induced differential expression of *IRF2* at rs13149699 as the driver of the trans network. IPA of rs13149699 trans-associated genes supported their modulation by IRF2 and interferon signaling, with overrepresentation of interferon-associated pathways and IRF2 identified as an upstream regulator ($P = 7.9 \times 10^{-11}$).

To investigate whether these trans-associated genes were bona fide IRF2 targets in monocytes, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) for primary monocytes from two healthy volunteers using the same experimental conditions to define IRF2 binding sites (table S5). We found that genes in trans to rs13149699 were significantly enriched for IRF2 binding in

corresponding treatments (Fig. 4, F to H) with 8.0- and 8.7-fold relative increase in trans-associated genes residing in a 25-kb window around IRF2 binding sites after 2-hour LPS or IFN- γ , respectively, compared to those not in trans to rs13149699 (LPS2 $P = 3.5 \times 10^{-6}$, IFN- γ $P = 1.9 \times 10^{-6}$; Fisher's exact test). It was apparent that not only were genes in trans to rs13149699 dependent on stimulation, but also that different stimuli elicited different genes in trans, implying that IRF2 regulation is similarly context-

dependent. IRF2 is induced by IFN- γ and has both transcriptionally repressive and activating properties, antagonizing the action of IRF1 and modulating the IFN-induced immune response, notably to viral infection (31–33). Consistent with this, whereas most genes in trans to rs13149699 showed opposite allelic association to the cis-eQTL for *IRF2*, indicating inhibition by IRF2 expression, a significant proportion of trans genes showed the same allelic association consistent with induction by higher IRF2

expression (fig. S10). This analysis highlights how ChIP-seq can confirm putative mechanisms of trans associations and reveal context-specific transcription factor targeting of genes.

An Inducible Trans Gene Network Involving *NFE2L3* and the Proteasome

A further example of a context-specific trans network involving a transcription factor consisted of 23 genes encoding proteins involved in the ubiquitin-proteasome system (34), whose

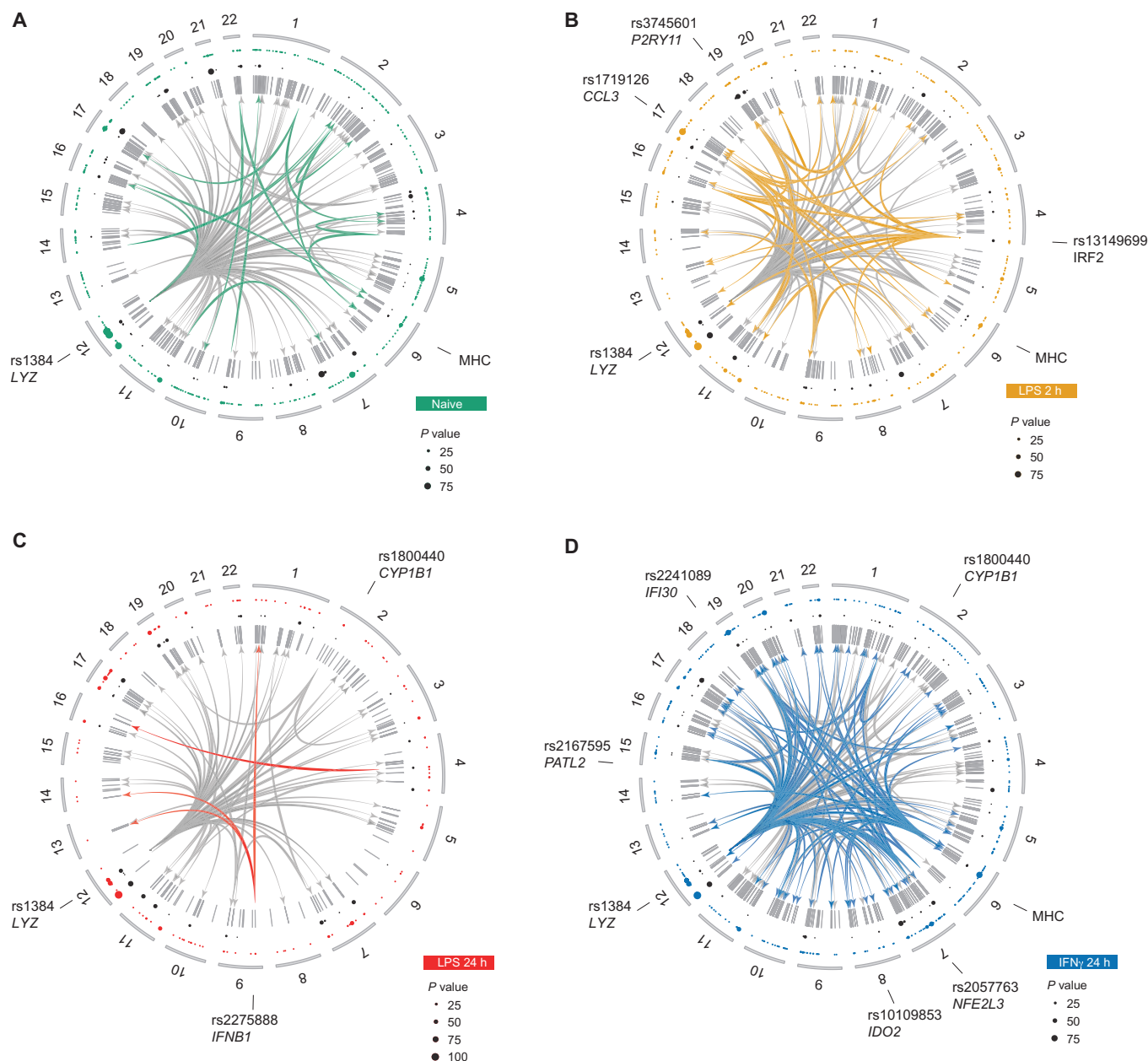


Fig. 2. Trans-eQTL demonstrate context specificity and identify master regulatory loci after treatment. (A to D) Circular plots demonstrating chromosomal location of trans-acting eQTL of naïve (A), 2-hour LPS treatment (B), 24-hour LPS treatment (C), and IFN treatment (D). From outside to inside: examples of major trans hubs are indicated by rs number of lead eSNP and for that eSNP the name of any cis gene, the nearest gene or genomic region; chromosome number; colored dots are significant trans-eQTL (color denotes treatment, size relative $-\log_{10}$

P value as per legend); black dots are significant cis-eQTL to the same SNP in these treatments, possibly identifying driver genes; gray lines indicate probes significantly regulated in trans; innermost joining lines illustrate start and end points of multiloci trans-eQTL (unique to a treatment by colored lines, observed in a minimum of two data sets by gray lines)—only multiloci eQTL mapping to more than one probe with an FDR < 0.05 in the shared data set ($n = 228$) are illustrated.

expression was associated with rs2057763 after IFN- γ (fig. S11 and table S4). Twelve of these trans genes encode subunits of the 26S proteasome, including the 20S proteasome core (*PSMA1*, *PSMB2*, *PSMB4*, and *PSMB6*) together with the 19S regulator complex (*PSMC3*, *PSMC4*, *PSMC5*, *PSMD1*, *PSMD3*, *PSMD11*, *PSMD13*, and *PSMD14*), suggesting that this SNP alters the expression of a key proteasomal regulator in response to IFN- γ . IPA revealed that protein

ubiquitination was the only significant pathway ($P = 9 \times 10^{-20}$) with two top regulators of this network: *NFE2L2* ($P = 5.4 \times 10^{-14}$), encoding Nrf2, a transcription factor that regulates genes containing antioxidant response elements (ARE) in their promoters, and the known Nrf2 inducer molecule 1,2-dithio-3-thione. rs2057763 is an intergenic SNP 36.7 kb from the closest gene on chromosome 7p15 encoding *NFE2L3*, a relatively poorly character-

ized *NFE2L2* homolog, recently recognized to play a key role in inflammation (35). Probes to *NFE2L3* were not analyzed in the primary analysis because of risk of SNP hybridization artifacts. We therefore performed quantitative polymerase chain reaction (qPCR) on both the 3' untranslated region and exons 1 and 2 of *NFE2L3* in monocytes from a randomly selected subset of 84 individuals after IFN- γ and observed a cis-eQTL for rs2057763 (fig. S11),

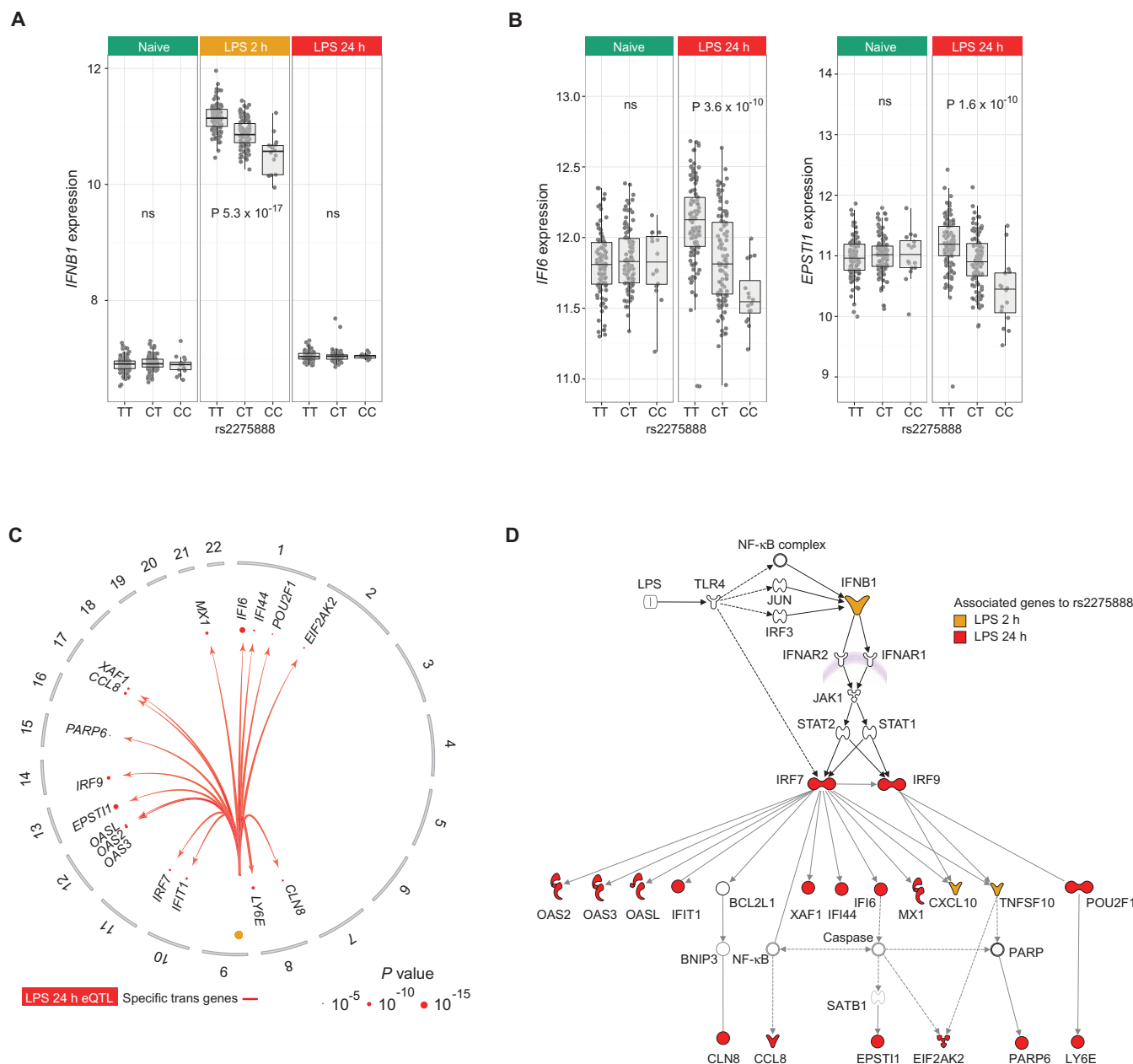


Fig. 3. Temporal effects for a stimulus-specific trans-eQTL. (A) A stimulus-specific local cis-acting eQTL for *IFNB1* in monocytes after 2-hour LPS treatment tagged by rs2275888, which is in complete linkage disequilibrium ($r^2 = 1$) with 15 other SNPs located ~60 kb telomeric to *IFNB1* within the intronic sequence of *PTPLAD2*. (B) rs2275888 is significantly associated in trans with expression of known interferon-induced genes including *IFI6* and *EPSTI1* only after 24-hour LPS. (C) Single SNP analysis at rs2275888 to resolve further genes in trans defined 17 genes

showing significant trans association (FDR < 0.05) to rs2275888 after 24-hour LPS stimulation as illustrated in this circular plot. (D) IPA network analysis of trans-associated genes after LPS induction revealed a major network containing 19 genes ($P = 1 \times 10^{-44}$) based on the fit of the trans genes and biological functions. The IFN β signaling cascade is shown with trans-associated genes to rs2275888 highlighted supporting the associations arising through cis-eQTL-driven differential expression of *IFNB1*.

supporting *NFE2L3* as the probable driver behind this network.

Informativeness for Disease

To further investigate the relationship between innate immune stimuli-induced eQTL and human disease risk loci resolved by GWAS, we first defined independent associations to the same probe interrogating gene expression within and between treatments. To do this, we resolved SNPs associated with the same eQTL into independent peaks of association among the shared data set of 228 individuals by conditional analysis (17). This revealed that although the majority of probes associated with a single peak, 9.8% of probes had a second peak in naïve monocytes, with up to three peaks in a small number of instances (Fig. 5A). Similar proportions were observed after treatment, the number of probes with two peaks modestly increasing to 11% after IFN- γ treatment. Considering all probes with a peak in the naïve state, additional peaks specific to stimulation were found, most markedly after IFN- γ treatment (Fig. 5B).

The overlap between primary peaks defined for each condition and disease loci reported in the GWAS catalog (www.genome.gov/gwastudies/) was subsequently assessed. Overall, 467 primary peaks overlapped with a GWAS locus, 248 of which were only observed in a stimulated state (table S6). To ascertain whether specific traits were enriched, we curated GWAS traits for membership of one or more disease categories on an organ-specific and systems approach. Observed cis-eQTL were found to be most significantly overrepresented in traits related to bacterial infection (naïve, 2-hour LPS), renal diseases (all except 2-hour LPS), and inflammation and immunity (all subsets) (Fig. 5C). Correspondingly, we observed underrepresentation in cancer, cardiovascular, and genetic traits.

The observation that many GWAS loci overlapped with eQTL specific to induced cells (Fig. 5D) is consistent with the hypothesis that disease risk alleles may manifest activity in a context-specific manner (36, 37). Noteworthy examples we identified include a unique cis-eQTL to the serine threonine kinase gene *ATM* after exposure to IFN γ (rs609261, $P = 7.8 \times 10^{-89}$) in complete LD to a GWAS SNP for metformin response in diabetes (38). Notably, IFN- γ has been shown to activate the ATM pathway (39), and ATM itself plays a key role in the IFN- γ response (40). Our data suggest that differential expression of *ATM* may be driving this association with metformin response in a highly context-specific manner.

For *IRF8*, a novel treatment-specific cis-eQTL is seen, maximal after induction of gene expression by LPS for 2 hours ($P = 9.9 \times 10^{-19}$), for which the peak SNP, rs17445836, is also a lead GWAS SNP for multiple sclerosis (MS) and associated with up-regulation of interferon response gene sets in peripheral blood mononuclear cells (PBMCs) from MS patients (41). For the chemokine receptor 3 gene (*CCR3*), we

also find evidence of a stimulus-specific cis-eQTL, after 24-hour LPS ($P = 2.3 \times 10^{-14}$), for which the lead eSNP was recently reported as the peak GWAS SNP for Behçet's disease, a multisystem

inflammatory disorder involving severe vasculitis (42). Other observations include an eQTL to *IRF1* with the peak SNP ($P_{LPS2} = 6.9 \times 10^{-17}$) overlapping with a Crohn's locus (43) and an eQTL

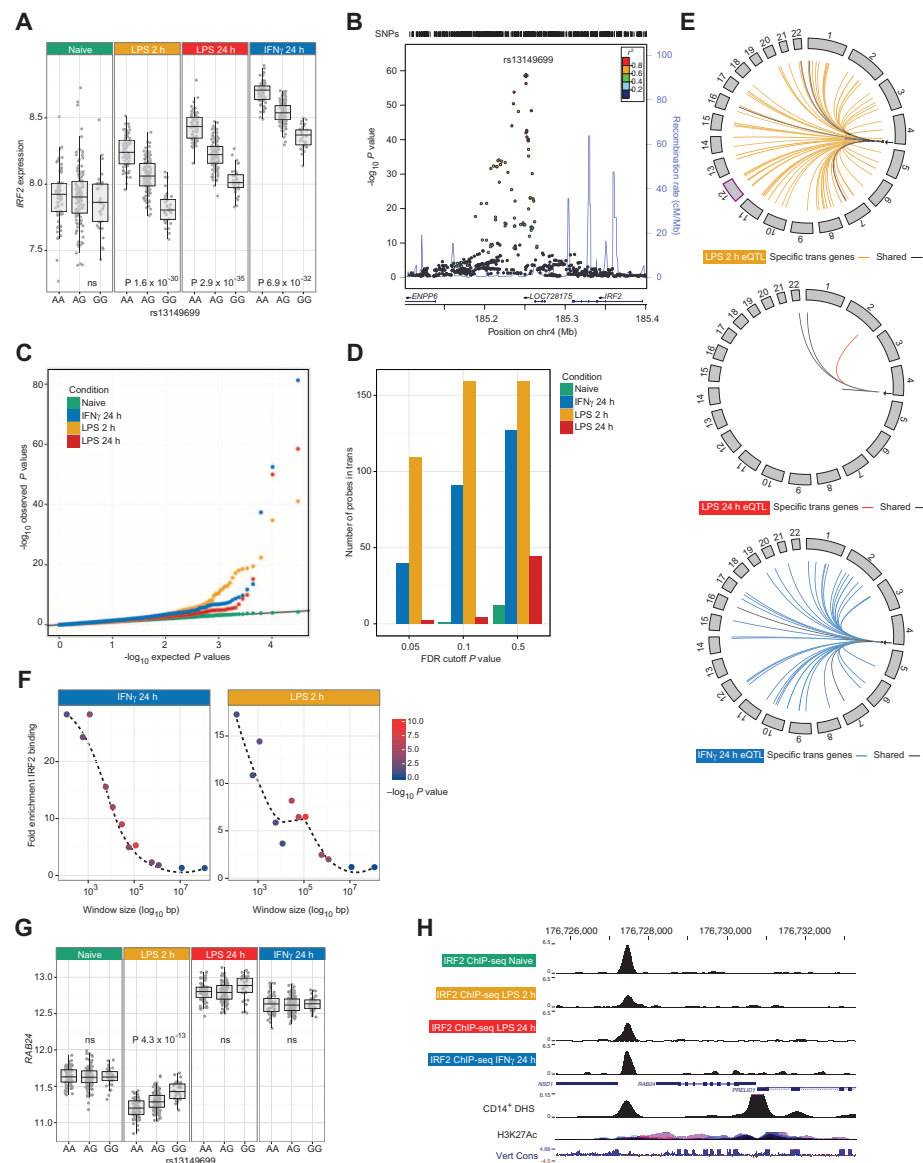


Fig. 4. Cis regulation of *IRF2* at rs13149699 has profound transcriptional consequences in trans. (A) A significant cis-eQTL to the transcription factor *IRF2* seen only after treatment (P values shown for shared data set; in the full data sets, $P_{LPS2} = 2.1 \times 10^{-35}$, $P_{LPS24} = 6.4 \times 10^{-55}$, $P_{IFN\gamma} = 2.6 \times 10^{-59}$, and $P_{naïve} = ns$). (B) Local association plot for 367 individuals after IFN- γ treatment including imputed genotypes. The peak eQTL for *IRF2* remains rs13149699, 57-kb 3' to the gene. (C) eQTL analysis was performed on rs13149699 alone in the shared ($n = 228$) data set to identify associated genes missed due to correction for multiple testing across all SNPs—qq plots demonstrating number of probes with expression associated with rs13149699 across treatments. Treatment results in significant numbers of probes deviating from expected, with greatest deviation after 2-hour LPS. (D) Bar charts demonstrating number of significantly associated probes at different FDR thresholds. Note relative absence of associations in untreated state despite the same number of tests being performed. (E) Circular plots demonstrating location of trans-eQTL (FDR < 0.05) after analysis on rs13149699 in the complete data sets. (F) Windows flanking probes tested for eQTL were defined, and the number of *IRF2* ChIP-seq binding sites per treatment was counted. Probes in trans to rs13149699 were significantly enriched for *IRF2* binding in both treatments (Fisher's exact test). (G) Box plot showing allelic expression of *RAB24* by rs13149699. (H) ChIP-seq for *IRF2* from primary monocytes for different treatments illustrating differential binding 0.5-kb 3' to *RAB24*. Also shown are ENCODE tracks for CD14⁺ DNase I hypersensitivity, H3K27Ac (seven cell lines), and vertebrate conservation (Multiz Alignment & Conservation 100 Species).

range of diseases. Understanding the genetic underpinnings of interindividual variation in immune and inflammatory responses is therefore of fundamental importance. Here, we have assessed the effect of carefully defined innate immune stimuli of high relevance to monocyte activity upon the generation of primary monocyte eQTL, contrasting observations to those in the naïve state. We illustrate how the majority of genes analyzed in monocytes demonstrate an eQTL under at least one condition. The stimuli examined act through well-annotated pathways, causing large-scale changes in gene expression, and are known to affect chromatin remodeling. These two factors together likely underpin the high degree of context specificity observed for induced eQTL. Our data demonstrate that genetic polymorphisms play a significant role in determining the transcriptional response to differing innate immune stimuli in human monocytes and that such effects may be context-specific.

The modulatory effects of genetic variation on gene expression are seen for specific genes dependent on particular stimuli and the kinetics of the response. We uncover that such genetic effects may only be seen in the early response after induction of expression or less commonly in modulating the extent of subsequent down-regulation. Although we only analyzed two time points for LPS, it is plausible that similar temporal effects exist for IFN γ and other stimuli. We frequently observe pathways where multiple genes show individual and specific associations with particular genetic markers. Although we anticipated that eQTL might manifest only after cellular stimulation in highly inducible genes such as cytokines, as observed in *IL1B*, *IL6*, and *LTA*, less expected was the observation of similar induced eQTL in many upstream nodal genes such as *TRAF6* and *SOC31*.

The resolution of trans-acting genetic factors and associated gene networks is recognized to be highly informative but challenging (19, 28, 53). Treatment with innate immune stimuli reveals a number of marked trans-eQTL including regulatory hubs. This may reflect a synchronizing influence of stimulation on the expression patterns of monocytes, allowing underlying genetic influences to be defined by reducing stochastic variation at the individual cell level. Notably, we observe opposing quantitative effects of chronic LPS and IFN γ on the numbers of trans-associated genes to the MHC class II region that parallel underlying class II expression. These trans-eQTL are likely to arise from cis effects in the MHC and/or to specific human leukocyte antigen (HLA) alleles; however, elucidating the mechanisms underpinning this observation requires further investigation. Context-specific differential cis effects at other loci similarly are perceived to induce consequential trans networks as illustrated by those observed at *IFNB1* and *IRF2*. These data support a model where identifying the genetic effects on a system requires the identification of a pathway, and any relevant effects of variation

on upstream activation before the underlying impact of genetic variation and trans associations can be revealed.

The integration of mapping cis- and trans-acting loci for specific cellular contexts with comparable data sets defining transcription

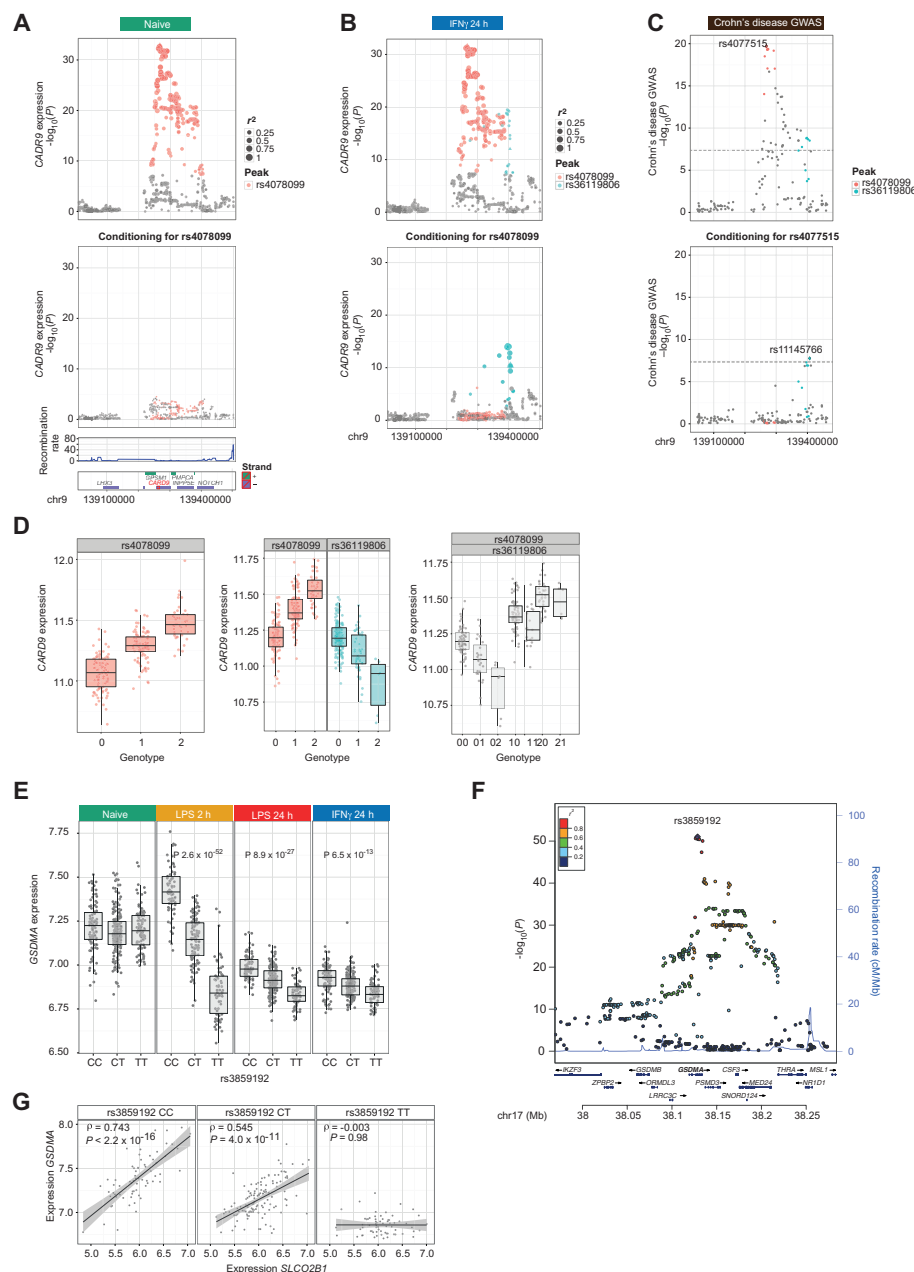


Fig. 6. Examples of context-specific eQTL informative for disease risk. (A) Local association plot showing evidence of cis-eQTL for *CARD9* in naïve cells. After conditioning for rs4078099, no evidence of association is seen. (B) After induction with IFN γ , in addition to the eQTL tagged by rs4078099, a second stimulus-specific independent peak of association is tagged by rs36119806. (C) Local association plots for CD with SNPs colored as per (A) and (B). The lead SNP rs40771515 is in high LD with rs4078099 ($r^2 = 0.93$); after conditioning, a second independent GWAS peak is seen (lead SNP rs11145766, $r^2 = 0.84$ with rs36119806). (D) Box plots showing expression of *CARD9* by allele in naïve cells, and after IFN γ stimulation, showing allelic association by SNP corrected for the effect of the other eQTL or by combination of inherited alleles for rs4078099 and rs36119806. (E) rs3859192 is the peak eQTL for expression of *GSDMA*, a transforming growth factor- β -regulated proapoptotic gene. (F) Local association plot for *GSDMA* expression after 2-hour LPS using imputed genotyping. (G) Allele-specific correlation for expression values per genotype demonstrates significant regulation of *GSDMA* expression in homozygous carriers of the C allele by the solute transporter *SLC02B1* after 2-hour LPS with no association being present in homozygous carriers of the T allele. Similar results were obtained analyzing monocytes after 24-hour LPS, and with both probes to *SLC02B1*, with no association observed in the naïve state.

factor binding, chromatin accessibility, and DNA looping across the genome (30) is a key step in resolving the functional genomic landscape on an individual basis. This study demonstrates the importance of considering the cellular context, and by extension a myriad of other stimuli and the developmental state, in the continued effort to understand the nature and functional consequences of genetic variation and translate this into patient benefit through individualized therapy and more rational drug design.

Materials and Methods

Volunteer Collection and PBMC Purification

This study was approved by the Oxfordshire Research Ethics Committee (COREC reference 06/Q1605/55). A total of 432 volunteers of European ancestry were recruited in the Oxfordshire area after written informed consent. The cohort consisted of 194 males and 238 females with a median age of 30 years (mean, 33 years; interquartile range, 19 to 56 years). Whole blood was collected into anticoagulant EDTA-containing blood collection tubes (Vacutainer System, Becton Dickinson). PBMCs, purified using Ficoll-Paque density gradients from 50 ml of whole blood, were washed three times in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (Invitrogen), and the number of cells was determined using a hemocytometer.

Monocyte Separation

Magnetic-activated cell sorting (MACS, Miltenyi) was performed to positively separate CD14^+ monocytes from 20 million PBMCs, as per the manufacturer's instructions. This is designed to provide a sample of ~99% purity. Flow cytometry was performed on a subset of samples with similar purities observed. Typical monocyte yields ranged from 1×10^6 to 10×10^6 monocytes per individual, of which 5×10^5 untreated cells were immediately suspended in RLT reagent (Qiagen) to form the naïve subset and snap-frozen for RNA extraction at a later date. All other monocytes were resuspended at a concentration of $1 \times 10^6/\text{ml}$ in 400 μl of prewarmed RPMI 1640 complete medium, supplemented with 20% fetal calf serum, penicillin/streptomycin, and L-glutamine. Cells were rested overnight (16 hours) at 37°C , 5% CO_2 in 5-ml nonadherent polypropylene cell culture tubes (BD Biosciences) before stimulation assays.

Stimulation Assays

For stimulation assays, 4×10^5 monocytes were exposed to ultrapure LPS (20 ng/ml) (catalog # tlr1-pelps, Invivogen) for either 2 or 24 hours. Alternatively, monocytes were exposed to IFN γ (catalog # 285-IF, R&D Systems) at a concentration of 20 ng/ml for 24 hours. The number of treated samples per individual depended on the total number of cells purified, and the priority on sample accrual was collation of IFN γ -treated followed by 24-hour LPS and then 2-hour LPS,

respectively. Experiments were terminated simultaneously, ensuring identical incubation periods for all samples. A subset of samples was kept in the incubator without stimulation throughout this period to ascertain incubator effects. All experiments were completed within 48 hours of blood sample collection.

Genomic DNA Extraction

Genomic DNA was extracted from whole blood using the Gentra Puregene Blood Kit following the manufacturer's instruction (Qiagen). The DNA was quantified by the PicoGreen dsDNA (double-stranded DNA) quantification assay (Invitrogen).

RNA Extraction

Total RNA was extracted in batches using the RNeasy Mini Kit from cells collected in the RLT reagent following the manufacturer's instruction using an additional DNase step to minimize contamination with genomic DNA (Qiagen). Total RNA was quantified by NanoDrop and Bioanalyzer for a subset of samples (Bioanalyzer RNA 6000 Nano Kit, Agilent).

Genotyping

Genotyping was performed with Illumina HumanOmniExpress-12v1.0 BeadChip with coverage of 733,202 separate markers. PLINK (34) was used for initial genotyping quality control (QC), including identity by descent (IBD) analysis to ensure individuals were unrelated, and heterozygosity testing and multidimensional scaling to identify population outliers compared to HapMap populations. For SNP filtering, we used sample and SNP call rates $>98\%$. We used a MAF of 4% and a Hardy-Weinberg equilibrium threshold set at 1×10^{-6} . We removed 11 individuals because of failure in one or more QC criteria, resulting in 421 individuals for further analysis with genotyping data available at 609,704 SNPs. Genotyping files were subsequently converted from PLINK output for use in MatrixEQTL.

Gene Expression Analysis

Total RNA from naïve and, where available, treated monocytes from each individual was quantified using the Illumina HumanHT-12 v4 BeadChip gene expression array platform with 47,231 probes. Of these, 28,688 probes correspond to coding transcripts with well-established annotations (RefSeq content NM), 11,121 to coding transcript with provisional annotation (XM), 1752 to noncoding transcript with well-established annotation (NR), 2209 to noncoding transcript with provisional annotation (XR), and 3461 to experimentally confirmed mRNA sequences that align to expressed sequence tag (EST) clusters from UniGene. Analysis of 1488 arrays was performed in three separate batches, the first consisting of 288 naïve monocyte samples as previously described (8). The second batch consisted of 636 samples from the same individuals randomized to individual and treat-

ment across array. A third batch consisted of 564 samples composed of 144 further naïve monocyte samples, as well as 420 treated samples that were similarly randomized to individual and treatment across arrays. Complementary RNA synthesis, cleanup, and hybridization were as per the manufacturer's instructions. Arrays were read, and data were exported using BeadStudio software (Illumina). Samples were subsequently processed in R using the R packages lumi, limma, and ComBat (55–57). Principal component analysis (PCA) was performed to identify samples within each treatment cohort with outlier expression for removal from further analyses. After QC and removal of duplicates and sample outliers, 1438 independent samples were suitable for further analysis. Raw data were transformed and normalized using robust spline normalization within lumi. Samples were then corrected for batch effects using ComBat. A linear model incorporating data from 59 untreated, incubated samples and 421 untreated, nonincubated samples was used to estimate the effect of incubation on gene expression. This was then regressed from all treated samples to minimize the influence of incubator effects on the analysis. Differential gene expression analysis was performed with the limma package. Pathway analysis was performed with IPA (Ingenuity Systems) to define gene networks, significant upstream regulators, and relationships with canonical signaling pathways. For network analysis of trans gene lists, a global molecular network based on the IP knowledge base (IPKB) was used with a P value based on the fit of the trans genes and list of biological functions within IPKB.

Probe Filtering

Normalization and correction for batch and incubator effects were carried out on all probes. Probes were subsequently selected for eQTL analysis. As previously described (8), probe sequences mapping to more than one genomic location using Basic Local Alignment Search Tool (BLAST) were excluded from eQTL analysis. Additionally, 6137 probes found to anneal in regions with SNPs present at a MAF of at least 1% in the European population of the 1000 Genomes Project were excluded from eQTL analysis to minimize potential confounding effects, as were probes mapping to non-autosomal locations. Remaining probes were included in eQTL analysis if they were present (Illumina detection $P < 0.01$) in $>2.5\%$ of samples from a specific treatment or, alternatively, $>5\%$ of all samples. This resulted in 15,421 probes for analysis, mapping to 11,168 independent Entrez ID.

eQTL Analysis

To account for confounding variation in the gene expression data, we carried out PCA for each of the four conditions. For each set of eQTL analyses, the dominant PC were chosen for inclusion as covariates in the model such

that the number of significant associations was maximized across all conditions. This resulted in the use of 30 (cis analysis of 228 shared samples), 16 (trans analysis of 228 shared samples), 40 (cis analysis of full data sets), and 20 PC (trans analysis of full data sets) as shown in fig. S2. The eQTL analysis was performed with the R package MatrixEQTL (18) using an additive linear model. SNPs were included in the cis analysis if they were located within 1 Mb of the gene expression probe under consideration. Correspondingly, associations between SNPs and probes outside this window were deemed trans. FDRs reported for the resulting associations are as reported by MatrixEQTL. For single SNP eQTL analysis, the same procedure was carried out, but with all analysis performed on a single SNP.

ChIP-seq Analysis

Monocytes were purified from donor blood cones from two healthy volunteers, and 5×10^6 cells were used per stimulation assay ($n = 6$). Cells were either untreated or treated as per LPS2 and IFN γ stimulations for the eQTL analysis. At the end of the experiment, cells were cross-linked and pellets were frozen. ChIP was performed with cross-linked cells lysed on ice in a lysis buffer [140 mM NaCl, 50 mM Hepes (pH 7.5), 1 mM EDTA (pH 8), 0.5 mM EGTA (pH 8), 0.5% (v/v) NP-40, 0.25% (v/v) Triton X, and 10% glycerol]. Lysis buffer was supplemented with 1 $\times$ Complete Protease Inhibitor Cocktail (Roche), 0.5 mM phenylmethylsulfonyl fluoride (Sigma), pepstatin (10 μ g/ μ l) (Sigma), and leupeptin hemisulfate (10 μ g/ μ l) (Sigma) before usage. Nuclear pellets were collected after brief centrifugation and washed using a buffer [10 mM tris (pH 8), 1 mM EDTA (pH 8), 2% (v/v) Triton X, 0.2% (v/v) Na deoxycholate, 10% glycerol] similarly supplemented as described above. Sonication to fragment cross-linked chromatin was carried out using the Bioruptor (Diagenode) at high power over 12 cycles of 30 s each. Fragmented chromatin was incubated overnight with Dynabeads (Life Technologies) pre-coated with 10 μ g of anti-IRF2 antibody [IRF-2 (C-19): sc-498, Santa Cruz Biotechnology]. Progressive washing of the chromatin-Dynabead mixture was then carried out in 10 mM tris (pH 8), 1 mM EDTA (pH 8), 1% (v/v) NP-40, 0.7% (v/v) Na deoxycholate, 0.5 M LiCl, supplemented as previously but using only 0.5 $\times$ Complete Protease Inhibitor. Cross-links were reversed after an overnight incubation in elution buffer [10 mM tris (pH 8), 1 mM EDTA (pH 8), and 1% SDS]. Immunoprecipitated DNA was obtained using phenol-chloroform extraction and purified after precipitation in ethanol. DNA was quantified using the High Sensitivity Qubit system (Life Technologies), and the fragmentation profile was assessed using a DNA HS 2200 TapeStation chip (Agilent).

ChIP libraries were prepared using the NEBNext DNA Sample Prep Master Mix Set 1 according to the manufacturer's specifications, but with

the following amendments: end repair was carried out using 5 μ l of NEBNext End Repair Reaction Buffer and 0.5 μ l of NEBNext End Repair Enzyme Mix. A-tail was carried out using 5 μ l of NEBNext A-tail Reaction Buffer and 0.3 μ l of NEBNext Klenow Fragment (3'→5' exo-) Enzyme Mix. Ligation was carried out using 10 μ l of NEBNext Quick Ligation Reaction Buffer and 0.5 μ l of NEBNext Quick T4 DNA Ligase Enzyme Mix. The NEBNext adapters were diluted 100-fold, and the amount added was adjusted if less than 5 ng of ChIP material was available. No size selection was carried out. PCR was done using the following reaction mix with 18 cycles of PCR: DNA (36 μ l), 5 $\times$ Phusion buffer (10 μ l), custom paired end (PE) primer 1 (1 μ l), custom PE primer 2 (1 μ l), dNTP (deoxynucleotide triphosphate) mix (1.5 μ l), Phusion polymerase (0.5 μ l). The cleanup after amplification was done at 1:0.85 (AMPure beads/DNA). The concentration of each library was determined by real-time PCR using Agilent qPCR Library Quantification Kit and a MX3005P instrument (Agilent). Sequencing was performed on an Illumina HiSeq 2000 using 50-base pair paired-end reads.

Reads were mapped using Stampy (58), and peaks were called with MACS2 (59) using non-immunoprecipitated DNA as a control for enrichment. Analysis was performed with BEDTools (60). Only peaks found in both samples were used for comparison with trans-associated probes to rs13149699. We observed 481 independent peaks that overlapped by a minimum of 10% in the two biological replicates in the untreated state: 170 after 2-hour LPS2 and 154 after IFN γ stimulation. The number of peaks found within defined distances (as per Fig. 4F) of trans-associated probes was counted and compared to those found within the same distance of non-trans-associated probes that had been similarly tested for eQTL. A Fisher's exact test was performed to test significance of number of occurrences per genomic window flanking the trans-associated probes versus non-associated probes.

Imputation of Genotypes

Genotypes were prephased with SHAPEIT2 (61), and missing genotypes were imputed with IMPUTE2 (62) in 5-Mb chunks against the 1000 Genomes reference panel (63). Summary statistics for each chunk were obtained using SNPTEST (https://mathgen.stats.ox.ac.uk/genetics_software/snpctest/snpctest.html), and sites with an information score of less than 0.9 or significant departure from Hardy-Weinberg equilibrium ($P < 1 \times 10^{-3}$) were excluded from further analysis. Genotype probabilities for all remaining sites were converted into dosage estimates and formatted for use with MatrixEQTL.

Conditional Analysis

To investigate the presence of local associations in addition to the most significant SNP reported for each probe, we carried out a conditional analysis with imputed genotypes. To this end,

the cis-eQTL analysis was repeated for all gene expression probes that had produced an association with a P value of no more than 5×10^{-8} , conditioning on the initial association. If no additional independent eQTL are present, it is expected that this will not identify any additional significant associations. Conversely, any associations that are independent of the first one are expected to remain and may increase in significance. This process was iterated until no further significant associations remained ($P < 5 \times 10^{-8}$). To confirm the presence of an independent signal, this process was repeated for the second and third most significantly associated SNP for a given probe. Only examples where the secondary signal was present in all instances are reported.

The resulting eQTL were further characterized by identifying the list of SNPs associated with each of the peak eSNPs. To this end, all SNPs that are showing significant association with gene expression are assigned to the eSNP with which they have the highest r^2 . Peaks identified in this way were matched across conditions, and the list of SNPs observed in all treatments in which the peak is present is reported.

Overlap Between eQTL Signals and GWAS Catalog

All traits listed in the catalog of published GWAS (www.genome.gov/gwastudies/; date accessed 10 January 2013) were extracted and expertly curated into phenotypic groupings blind to GWAS or eSNP data. Groups were defined on the basis of organ specificity of a particular trait, disease process, or type. These included cardiovascular, respiratory, gastroenterological, urological, rheumatological, neurological, renal, endocrine, hematological, dermatological, bone, cancer, immunity and inflammation, autoimmune, allergy, genetic, viral infection, bacterial infection, parasitic disease, measurement, physiological, metabolic, chronic or degenerative disease, reproduction, and drug-related. We recognized that classification of a given trait was possible into multiple groups, and to capture this diversity, for each trait, we assigned trait membership into three possible groups.

A reported GWAS SNP was considered to coincide with an eQTL identified during the conditional analysis of cis associations if the GWAS SNP itself or any SNPs with $r^2 > 0.8$ with this SNP were part of one of the eQTL peaks ($P < 5 \times 10^{-8}$). Enrichment of peak eQTLs for individual GWAS categories, as well as overall enrichment of GWAS SNPs, was tested with Fisher's exact test by comparing the overlap obtained for peak eQTLs to that observed for all SNPs tested for cis associations. Publicly available summary statistics for a 200-kb window around *CARD9* from the latest CD GWAS meta-analysis (46) were extracted using the Ricipili tool (www.broadinstitute.org/mpg/ricipili/). LD calculations were performed with PLINK (54) and the phase 1 1000 Genomes data (63).

Approximate conditional analysis, based on summary statistics and LD properties, was carried out using the method described in (46).

Flow Cytometry

To validate the changes in class II HLA observed at transcriptomic level with IFN- γ and LPS treatment, we assessed class II expression on primary monocytes by multiparametric flow cytometry. Monocytes were stimulated, as described, before being washed and stained with violet viability dye (Invitrogen) as per the manufacturer's instructions. To reduce nonspecific staining, a three-step procedure adapted from a protocol shared by R. Apps (National Cancer Institute, National Institutes of Health) was used. Human immunoglobulin G (IgG) was added to each tube, followed by mouse anti-human antibodies directed against HLA-DP (clone B7/21, Abcam), HLA-DQ (clone SPV-L3, Abcam), HLA-DR (clone L243, BD Biosciences), or all class II alleles (clone Tu39 and SK10/SPV-L3 combined) or an isotype control. After 20 min, cells were washed and stained with sheep anti-mouse PE-labeled antibody (Sigma) and sheep IgG.

After a further 20 min, cells were washed and stained with mouse anti-human CD14-FITC (fluorescein isothiocyanate) (clone 61D3, eBioscience) and mouse IgG (Sigma). Cells were fixed with the paraformaldehyde containing Reagent A (Life Technologies), and data were acquired immediately on a FACSCanto (BD Biosciences) machine. A minimum of 10,000 gated monocytes were acquired for each sample. Fluorescence-minus-one controls were used to set gate thresholds.

Data Presentation

Graphs were generated using ggplot2 (64) and R base packages. Box plots of gene expression data show values corrected for confounding variance using gene expression PC as per eQTL analysis. Circular plots were created using the ggbio R package (65), and local association plots were generated with LocusZoom (66).

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Supplementary Materials
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Common Genetic Variants Modulate Pathogen-Sensing Responses in Human Dendritic Cells

Mark N. Lee *et al.*

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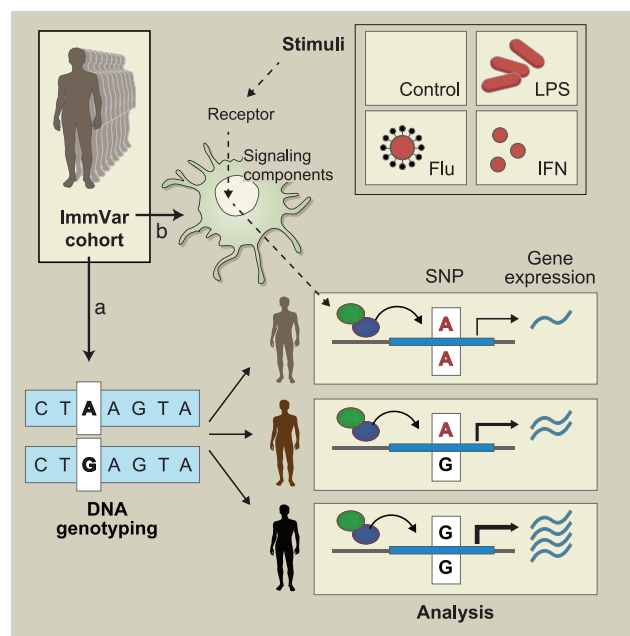
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Introduction: Variation in an individual's response to environmental factors is likely to influence susceptibility to complex human diseases. The genetic basis of such variation is poorly understood. Here, we identify natural genetic variants that underlie variation in the host innate immune response to infection and analyze the mechanisms by which such variants alter these responses.

Methods: We derived dendritic cells (DCs) from peripheral blood monocytes of healthy individuals (295 Caucasians, 122 African Americans, 117 East Asians) and stimulated them with *Escherichia coli* lipopolysaccharide (LPS), influenza virus, or the cytokine interferon- β (IFN- β) to generate 1598 transcriptional profiles. We genotyped each of these individuals at sites of common genetic variation and identified the genetic variants that best explain variation in gene expression and gene induction between individuals. We then tested mechanistic predictions from these associations using synthetic promoter constructs and genome engineering.

Results: We identified 264 loci containing genetic variants associated with variation in absolute gene expression in human DCs, of which 121 loci were associated with variation in the induction of gene expression by one or more stimuli. Fine-mapping identified candidate causal single-nucleotide polymorphisms (SNPs) associated with expression variance, and deeper functional experiments localized three of these SNPs to the binding sites of stimulus-activated transcription factors. We also identified a cis variant in the transcription factor, *IRF7*, associated in trans with the induction of a module of antiviral genes in response to influenza infection. Of the identified genetic variants, 35 were also associated with autoimmune or infectious disease loci found by genome-wide association studies.

Discussion: The genetic variants we uncover and the molecular basis for their action provide mechanistic explanations and principles for how the innate immune response to pathogens and cytokines varies across individuals. Our results also link disease-associated variants to specific immune pathways in DCs, which provides greater insight into mechanisms underlying complex human phenotypes. Extending our approach to many immune cell types and pathways will provide a global map linking human genetic variants to specific immunological processes.



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FIGURES IN THE FULL ARTICLE

Fig. 1. A strategy to identify gene-by-environment interactions in the innate immune responses of primary human DCs.

Fig. 2. Genome-wide expression profiles in MoDCs reveal response phenotypes.

Fig. 3. Association analysis reveals cis-eQTLs and cis-reQTLs.

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SUPPLEMENTARY MATERIALS

Materials and Methods
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RELATED ITEMS IN SCIENCE

Research Article
B. P. Fairfax *et al.*, *Science* **343**, 1246949 (2014).
Perspective
P. K. Gregersen, *Science* **343**, 1087 (2014).

Identifying the genetic basis of variability in the host response to pathogens. A cohort of 534 individuals donated blood for (a) genotyping of common DNA variants and (b) isolation of immune DCs. DCs were stimulated with viral and bacterial components, and the variability in individuals' gene expression responses was mapped to specific DNA variants, which were then shown to affect binding of particular transcription factors.

The list of author affiliations is available in the full article online.

*These authors contributed equally to this work.

†Corresponding author. E-mail: aregev@broad.mit.edu (A.R.); nhacohen@partners.org (N.H.)

Common Genetic Variants Modulate Pathogen-Sensing Responses in Human Dendritic Cells

Mark N. Lee,^{1,2,3*} Chun Ye,^{1*} Alexandra-Chloé Villani,^{1,2} Towfique Raj,^{1,2,4} Weibo Li,^{1,3} Thomas M. Eisenhaure,^{1,3} Selina H. Imboywa,² Portia I. Chipendo,² F. Ann Ran,^{1,5,6,7,8} Kamil Slowikowski,⁹ Lucas D. Ward,^{1,10} Khadir Raddassi,¹¹ Cristin McCabe,^{1,4} Michelle H. Lee,² Irene Y. Frohlich,² David A. Hafler,⁸ Manolis Kellis,^{1,10} Soumya Raychaudhuri,^{1,2,12,13} Feng Zhang,^{6,7,8} Barbara E. Stranger,^{14,15} Christophe O. Benoist,² Philip L. De Jager,^{1,2,4} Aviv Regev,^{1,16,17†} Nir Hacohen^{1,2,3†}

Little is known about how human genetic variation affects the responses to environmental stimuli in the context of complex diseases. Experimental and computational approaches were applied to determine the effects of genetic variation on the induction of pathogen-responsive genes in human dendritic cells. We identified 121 common genetic variants associated in cis with variation in expression responses to *Escherichia coli* lipopolysaccharide, influenza, or interferon- β (IFN- β). We localized and validated causal variants to binding sites of pathogen-activated STAT (signal transducer and activator of transcription) and IRF (IFN-regulatory factor) transcription factors. We also identified a common variant in *IRF7* that is associated in trans with type I IFN induction in response to influenza infection. Our results reveal common alleles that explain interindividual variation in pathogen sensing and provide functional annotation for genetic variants that alter susceptibility to inflammatory diseases.

Susceptibility to complex diseases depends on both genetic predisposition and exposure to environmental factors, with interactions between the two (G $\times$ E interactions) likely contributing substantially to disease risk (1, 2). However, the extent and mechanisms by which common human genetic variants interact

with the environment remain poorly explored and have been difficult to detect in clinical studies (1, 3). Genetic analysis of molecular traits, such as gene expression profiles, offers a promising way to dissect the molecular mechanisms underlying G $\times$ E interactions. Expression quantitative trait locus (eQTL) studies have been used to map genetic variants contributing to variation in gene expression, but have largely focused on steady-state expression in humans (4, 5) and thus have excluded G $\times$ E interactions. In model organisms, differences in growth conditions or treatment with various stimuli have revealed the existence of response eQTLs (reQTLs) (6–9), defined as QTLs associated with the change in expression after stimulation. Here, we sought to identify reQTLs in humans, to explain the mechanism by which the environment interacts with these variants, and to determine whether these variants are associated with immune-mediated diseases.

We used dendritic cells (DCs) of the innate immune system as a model system for reQTL studies, with physiological and clinical relevance. DCs play a direct role in the host recognition of pathogens using specialized sensors that engage well-characterized signaling and transcriptional networks. For example, bacterial lipopolysaccharide (LPS) activates two distinct arms of the Toll-like receptor 4 (TLR4) pathway, whereas influenza infection primarily activates the RNA-sensing TLRs (for example, TLR3) and the RIG-I-like receptors (for example, RIG-I) (10). These, in turn, lead to the translocation of transcription factors (TFs) from the cytoplasm into the nu-

cleus to induce the expression of immune genes, including interferon- β (IFN- β) secretion that engages the type I IFN response pathway to induce the expression of hundreds of antiviral effectors. Genetic studies have associated common variants near many genes in these pathways with risk of different inflammatory diseases (11, 12). DCs also play a critical role in the pathologic immune responses underlying inflammatory diseases (11–13), also reflected in recent genome-wide association studies (GWAS) of several diseases (14–17), especially inflammatory bowel disease (14).

Results

Assessing the Impact of Genetic Variation on Pathogen Sensing in Primary Human DCs

We developed an integrated experimental and computational pipeline (Fig. 1A) to identify variability in human DC responses and associate this variability with common genetic variants. First, we optimized a high-throughput protocol to isolate primary CD14⁺CD16^{lo} monocytes from human blood samples (fig. S1, A to F), differentiate them into monocyte-derived DCs (MoDCs), and stimulate them with three immunostimulatory agents (Fig. 1B): *Escherichia coli* LPS, influenza virus, or IFN- β (a cytokine induced by LPS and influenza). Second, we collected genome-wide transcript profiles from resting and stimulated DCs from 30 healthy individuals and computationally identified a “signature” of 415 genes that would be informative of variation in the response in a larger cohort. Third, we generated 1598 transcriptional profiles [using an amplification-free platform suitable for small cell numbers (18)] from DCs isolated from 534 healthy individuals (295 Caucasians, 122 African Americans, and 117 East Asians) (table S1) in four states: resting, LPS-stimulated, influenza-infected, and IFN- β -stimulated. Finally, we associated expression variation with genetic variation to identify cis- and trans-eQTLs and reQTLs, and we investigated candidate reQTLs for their mechanism of action.

Variability of Pathogen-Sensing Responses Between Individuals

To assess the interindividual variability of DC responses, we first profiled genome-wide expression using microarrays in resting, LPS-stimulated, and influenza-infected MoDCs isolated from each of 30 healthy individuals (18 Caucasians, 6 Asians, and 6 African Americans). We found 1413 genes that were regulated in LPS- or influenza-treated cells [$\log_2$ (fold change) >0.75 or ≤ -1.5 ; false discovery rate (FDR) <0.01 ; fig. S2A and table S2A; see table S2, B to I, for enriched biological processes] at 5 and 10 hours, respectively (time points selected to have maximal expression of induced clusters) (fig. S1, B and C).

We quantified reproducibility in these responses by recalling 12 of the 30 donors 2 to 9 months after the first collection for MoDC

¹Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard, Cambridge, MA 02142, USA. ²Harvard Medical School, Boston, MA 02115, USA. ³Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Charlestown, MA 02129, USA. ⁴Program in Translational NeuroPsychiatric Genomics, Department of Neurology, Brigham and Women’s Hospital, Boston, MA 02115, USA. ⁵Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. ⁶McGovern Institute for Brain Research, MIT, Cambridge, MA 02139, USA. ⁷Department of Brain and Cognitive Sciences, MIT, Cambridge, MA 02139, USA. ⁸Department of Biological Engineering, MIT, Cambridge, MA 02139, USA. ⁹Bioinformatics and Integrative Genomics, Graduate School of Arts and Sciences, Harvard University, Cambridge, MA 02138, USA. ¹⁰Computer Science and Artificial Intelligence Laboratory, MIT, Cambridge, MA 02139, USA. ¹¹Department of Neurology, Yale School of Medicine, New Haven, CT 06511, USA. ¹²Divisions of Genetics and Rheumatology, Department of Medicine, Brigham and Women’s Hospital, Boston, MA 02115, USA. ¹³Arthritis Research UK Epidemiology Unit, Musculoskeletal Research Group, University of Manchester, Manchester Academic Health Sciences Centre, Manchester M13 9NT, UK. ¹⁴Section of Genetic Medicine, The University of Chicago, Chicago, IL 60637, USA. ¹⁵Institute for Genomics and Systems Biology, The University of Chicago, Chicago, IL 60637, USA. ¹⁶Department of Biology, MIT, Cambridge, MA 02139, USA. ¹⁷Howard Hughes Medical Institute.

*These authors contributed equally to this work.

†Corresponding author. E-mail: aregev@broad.mit.edu (A.R.); nhacohen@partners.org (N.H.)

isolations and profiling (table S1); we identified genes whose expression interindividual variance is significantly higher than their intraindividual variance based on the serial replicates [taking into account known covariates including gender, age, and population and unknown factors using surrogate variable analysis (19)]. Two hundred twenty-two of the 1413 regulated genes (16%) showed significantly higher ($FDR < 0.1$) inter- than intraindividual variability, either in their absolute expression or in the differential expression (stimulated/baseline level) to at least one stimulus (Fig. 2A and table S3). These results suggest that there is consistent variation in these traits that may have a genetic basis.

Expression Profiling of a Pathogen Response Signature

Mapping the genetic basis of interindividual variation in pathogen responses requires profiling of DC gene expression in a larger cohort; this poses a substantial technical challenge given the limited numbers of primary cells and multiple stimuli. Furthermore, the responses of DCs to virus, bacterial ligands, and IFN are limited in scope and do not encompass the entire genome. We therefore defined a 415-gene signature set (Fig. 2B and table S3) that could be monitored in small numbers of cells using a sensitive multiplex RNA detection system (18), allowing us to scale up our study. The signature consisted of (i) all 222 of 1413 regulated genes with greater inter- than intraindividual variability in the microarray study; (ii) all 24 regulated genes exhibiting greater inter- than intrapopulation

variability ($FDR < 0.1$) (table S3) in the microarray study; (iii) 76 genes comprising the known components of the TLR4, TLR3, RIG-I, and IFNAR pathways (Fig. 1B) (10, 20–22); (iv) 61 regulated genes that play key roles in the DC response (for example, *IFNB1* and *IFITM3*) (22–24); (v) 28 regulated genes residing in loci previously associated with autoimmune or infectious diseases (25); and (vi) 35 control genes including those that had among the lowest interindividual variance in the microarray profiles. This representative signature allowed us to monitor genes with high interindividual variability, as well as key components and responses of the pathogen-sensing pathway.

We then generated 1598 transcriptional profiles, using the 415-gene signature, from MoDCs isolated from 534 healthy individuals (and 37 serially collected replicates) in up to four conditions: resting (528 individuals), LPS-stimulated (356), influenza-infected (342), and IFN- β -stimulated (284) (Fig. 2C; fig. S1, E and F; and table S1). The IFN- β stimulus allowed us to partition genes that were induced by both LPS and influenza (cluster III) into those induced secondary to type I IFN signaling (cluster IIIa) or by other mechanisms shared between these bacterial and viral sensing pathways (cluster IIIb), such as nuclear factor κ B (NF- κ B) or activating protein 1 (AP-1) activation (Figs. 1B and 2C). The signature expression profiles clustered similarly to those from the microarray analysis (Fig. 2C and fig. S2C) and captured most of the variance in the genome-wide profile [cross-validation, >0.99 ground-truth Pearson correlation coefficient; fig. S2D (26)],

demonstrating the utility of the signature to capture the genome-wide response.

reQTLs That Modulate Cellular Responses to Pathogens

We mapped cis-eQTLs and cis-reQTLs by testing for association between common single-nucleotide polymorphisms (SNPs) (minor allele frequency $>5\%$; genotyped with Illumina HumanOmniExpress BeadChip) and variation in either absolute transcript abundance (eQTL) or stimulation-induced change in transcript abundance (reQTL) in a nearby gene (in which the transcriptional start site or stop codon is within 1 Mb of the SNP). We pooled individuals from the three human populations together and included covariates and principal components to increase statistical power while avoiding systematic confounders such as population structure and expression heterogeneity. We identified 264 genes with cis-eQTLs in at least one condition (permutation $FDR < 0.05$) (Fig. 3A; fig. S3, A to C; and table S4, A to D) (27). Notably, 22 of 264 genes also associated with additional independent cis-eQTLs after conditioning on the top five most significant SNPs in each cis-eQTL region (table S6 and fig. S3D) (27), which reflects the complexity of the regulatory landscape around each gene.

We detected 121 cis-reQTLs (Fig. 3B and table S4, E to G; permutation $FDR < 0.05$; 91% internal reproducibility in at least two human populations, table S5), the subset of genetic variants that affect the induction of gene expression by LPS, influenza, or IFN- β . Of the 121 genes with cis-reQTLs, 7 associations were found

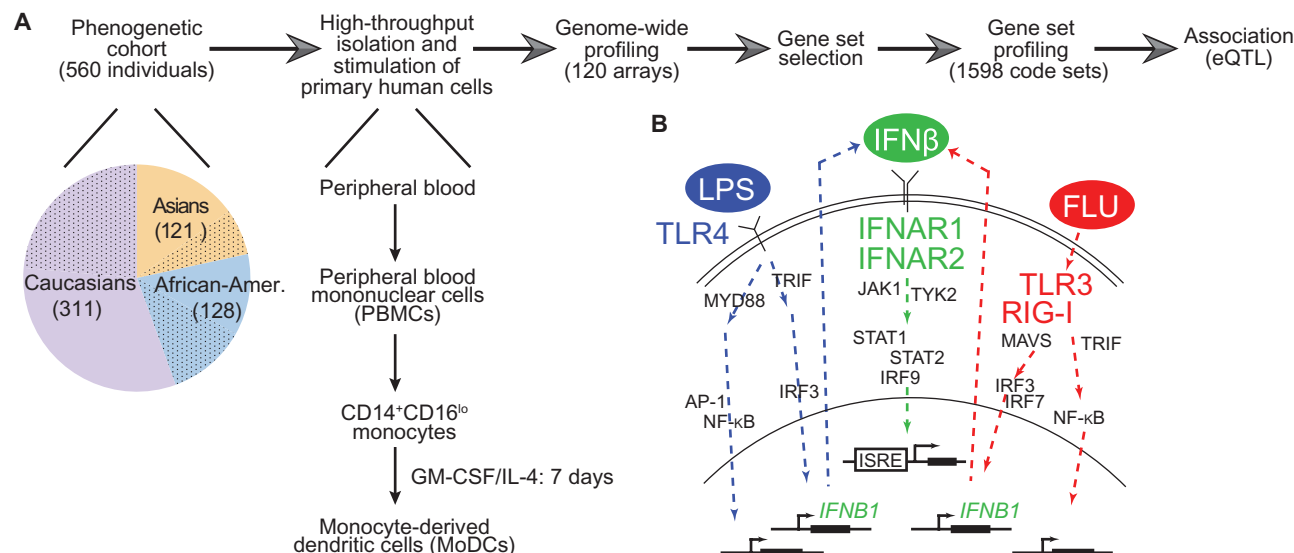


Fig. 1. A strategy to identify gene-by-environment interactions in the innate immune responses of primary human DCs. (A) Strategy used to identify baseline and response eQTLs and reQTLs, consisting of five steps: (i) high-throughput isolation and stimulation of primary human MoDCs from 560 healthy individuals (dotted slices, male; solid-colored slices, female) collected as part of the PhenoGenetic cohort; (ii) whole-genome gene expression measurements in a subset of the cohort; (iii) selection of signature gene set, consisting of regulators and regulated genes; (iv) digital multiplex gene expression measurements of signature genes in the entire cohort; and (v) mapping of genetic variation to

expression variation. GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4. **(B)** Model of innate immune pathways activated by three stimuli demonstrating their downstream relationships. LPS from *E. coli* engages the TLR4 receptor; IFN- β engages the heterodimeric IFNAR receptor; influenza A/PR8 (Δ NS1) ("FLU") engages the cytoplasmic TLR3 and RIG-I receptors. Receptor engagement activates signal transduction cascades that regulate expression of inflammatory genes, IFNs, and IFN-stimulated genes. IFNAR activation also occurs during LPS and FLU stimulations because LPS and FLU both induce IFN production, leading to activation of ISREs. JAK1, Janus kinase 1.

only in the influenza condition (for example, *IFNA21*; Fig. 3C and fig. S3B; meta-analysis, $P < 1 \times 10^{-5}$; permutation FDR = 0.02 to 0.03); 15 in both the LPS and influenza conditions (for example, *TEC*; Fig. 3C and fig. S3B); and 57 in all three stimulation conditions (for example, *ARL5B*, *SLFN5*, and *CLEC4F*; Fig. 3, C to E,

and fig. S3B), likely reflecting activation of the shared IFN- β pathway. We hypothesized that causal variants driving cis-reQTLs alter the sequence of genomic elements that respond to TFs downstream of the pathogen-sensing receptors (Fig. 3F), and represent gene-by-environment interactions.

To validate cis-reQTLs, we quantified allele-specific expression for pan-stimulation-specific associations in heterozygote individuals (Fig. 3, D and E). This was feasible for a gene with an exonic SNP (*CLEC4F* rs2075221) that was the most significant reQTL SNP, and for a gene with an exonic SNP (*SLFN5* rs11651240) in linkage

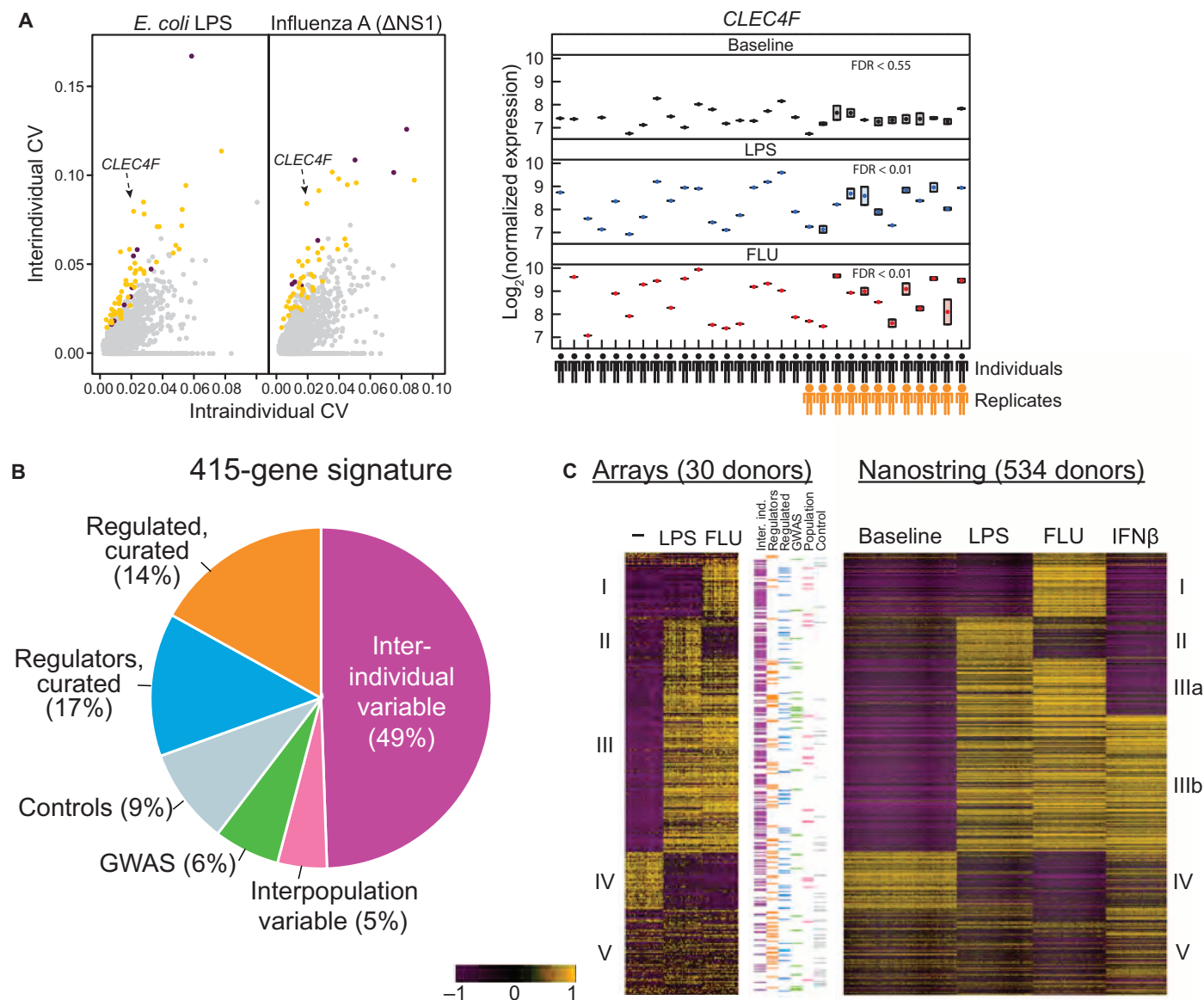


Fig. 2. Genome-wide expression profiles in MoDCs reveal response phenotypes. (A) Coefficient of variation (CV) of gene expression between 30 different donors ("Interindividual CV") plotted against CV of expression within 12 serial replicate samples ("Intraindividual CV") for each differentially regulated (fold change >0.75 or <-1.5) gene after LPS or FLU stimulation. Yellow (up-regulated) and purple (down-regulated) circles represent genes with significant (moderated t test, FDR < 0.1) inter- versus intraindividual variation. (Right) log₂(expression, microarray data) of *CLEC4F* in baseline, LPS-stimulated, and FLU-infected MoDCs from 30 donors and 12 replicates, demonstrating example of a gene that shows significant (FDR < 0.01) inter- versus intraindividual variation after LPS and FLU stimulations but not at baseline (fig. S2B). Standard error of replicate samples ($n = 12$) is shown for each sample. (B) Pie chart of 415 signature genes selected for Nanostring code set: 222 (49%) are regulated genes that showed significant (mixed model variance components test, permutation FDR < 0.1)

inter- versus intraindividual variability; 61 (14%) are curated, regulated genes with a known function in the innate immune response; 76 (17%) are curated regulators in the TLR4, TLR3, RIG-I, and IFNAR pathways; 41 (9%) are control genes including low-variance genes, sex-specific genes, and nonexpressed genes; 28 (6%) are regulated genes that were reported in the regions of autoimmune and infectious disease GWAS; and 21 (5%) are regulated genes that showed significant inter- versus intrapopulation variability. (C) Gene expression heat map of the 415-gene signature in MoDCs from the microarray study (30 individuals) and the Nanostring study (534 individuals). Each row represents a gene; each column represents a donor sample at baseline, stimulated with LPS, infected with FLU, or stimulated with IFN- β . Rows were clustered by k -means clustering of Nanostring data set with major clusters (I, II, IIIa, IIIb, and IV) labeled. Between the two heat maps, each row was labeled with colored dashes corresponding to one of the six categories described in (B).

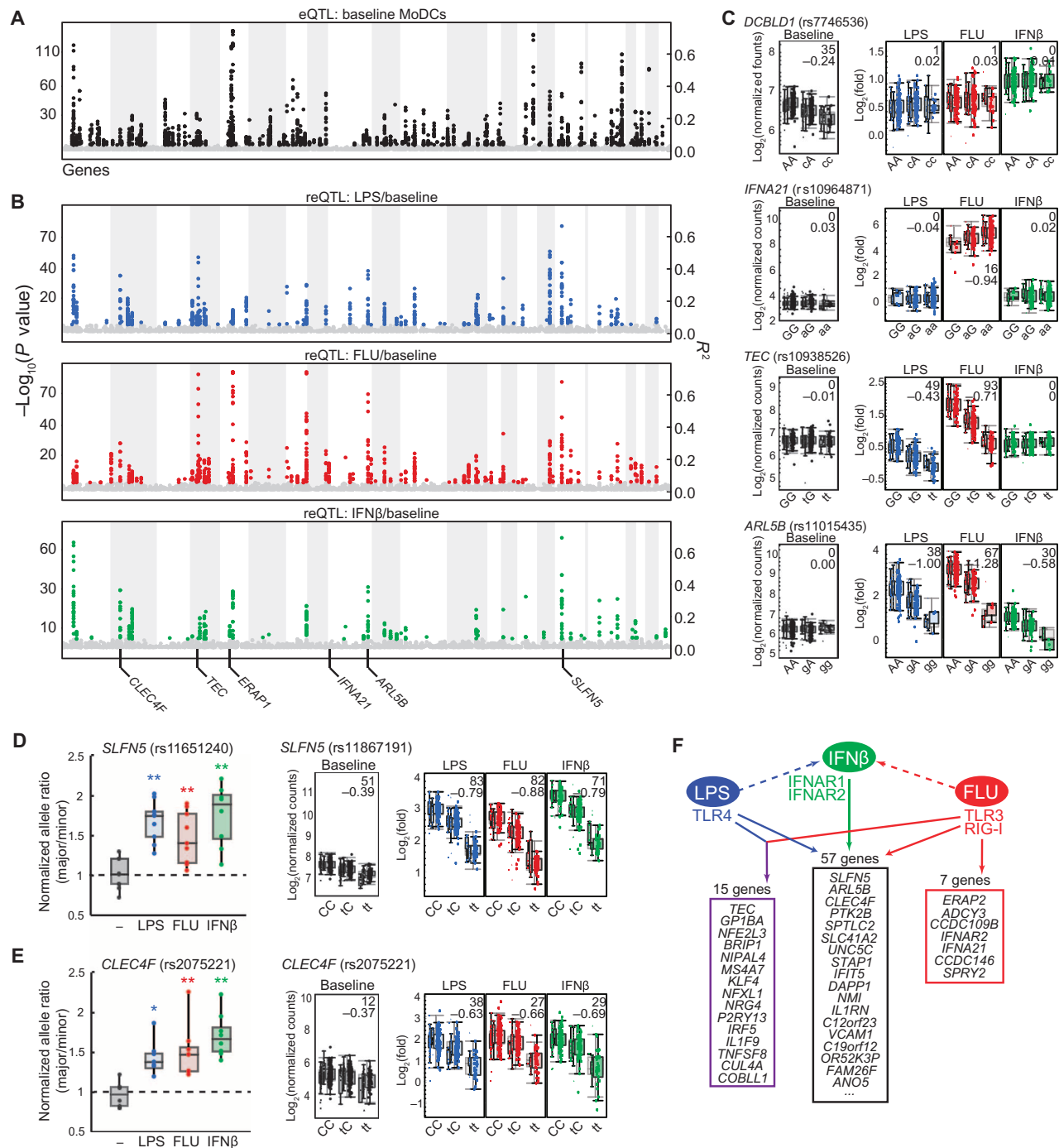


Fig. 3. Association analysis reveals cis-eQTLs and cis-reQTLs. (A and B) Manhattan plot of cis-eQTLs (A) (baseline expression) and cis-reQTLs (B) (LPS-, FLU-, and IFN-β-stimulated fold changes relative to baseline) showing $-\log_{10}(P)$ values (left y axis) and R^2 values (right y axis) for all cis-SNPs, displayed on the x axis with associated genes ordered by chromosomal location. (C) Box-whisker plots showing expression [left; $\log_2(\text{nCounts})$, y axis] or fold change [right; $\log_2(\text{fold})$, y axis] of *DCBLD1*, *IFNA21*, *TEC*, and *ARL5B* in resting, LPS-stimulated, FLU-infected, and IFN-β-stimulated MoDCs as a function of genotype of the respective cis-SNPs (x axis: rs27434, rs10964871, rs10938526, and rs11015435). African Americans, Asians, and Europeans in this order are displayed as separate box-whisker plots adjacent to each other in each condition. $-\log_{10}(P)$ values and β statistics are displayed in top right corners. (D and E) Allelic imbalance analysis of *SLFN5* (D) and *CLEC4F* (E) in resting, LPS-stimulated, FLU-infected, and IFN-β-stimulated MoDCs, showing the ratio of gene expression between the major and

minor alleles in heterozygote (rs11651240 for *SLFN5*, rs2075221 for *CLEC4F*) cDNA samples [$n = 8$ in (D); $n = 9$ in (E)] normalized to the ratio in the corresponding genomic DNA samples; significant deviation from 1.0 (dashed line) is consistent with allelic imbalance. Data are from one experiment representative of three (mean and SD shown). $*P < 0.01$, $**P < 0.001$, compared to unstimulated cells (Student's t test). On the right panels, box-whisker plots showing expression [left; $\log_2(\text{nCounts})$, y axis] or fold change [right; $\log_2(\text{fold})$, y axis] of *SLFN5* (D) and *CLEC4F* (E) in resting, LPS-stimulated, FLU-infected, and IFN-β-stimulated MoDCs as a function of genotype of the respective cis-SNPs: rs11867191 and rs2075221. (F) Schematic showing the different combinations of stimuli leading to significant cis-reQTLs, with the most significant examples listed. Specificity to conditions was defined with M value >0.9 taken as the inclusion criteria and M value <0.1 taken as the exclusion criteria for each condition.

disequilibrium with the most significant reQTL SNP (rs11867191, $R^2 = 0.501$ CEPH, CEU). As predicted, transcripts derived from the major and minor alleles differed in expression after stimulation with LPS (*SLFN5*: $P < 0.001$, t test; *CLEC4F*: $P < 0.01$); influenza (*SLFN5*: $P < 0.001$; *CLEC4F*: $P < 0.01$); or IFN- β (*SLFN5*: $P < 0.001$; *CLEC4F*: $P < 0.001$) but not at baseline (Fig. 3, D and E).

Cis-reQTLs That Alter the Sequence of TF Binding Sites

We hypothesized that the causal variants underlying cis-reQTLs functionally alter the chromosomal binding sites of TFs activated by one or more stimuli. To fine map reQTLs, we performed a trans-ethnic meta-analysis (28) of imputed variants in each population (~10 M). We then examined whether the most highly associated meta-reQTLs overlap with TF binding sites identified in high-throughput human chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq) data sets (for example, ENCODE) or

computationally predicted conserved regulatory elements (29–31). We found substantial enrichment (table S7) of known binding sites for TFs from the signal transducer and activator of transcription (STAT) family (TFs that are activated downstream of type I IFN signaling). The most significant enrichment over background was found for STAT2 (116-fold, binomial $P < 2.55 \times 10^{-21}$) and STAT1 (126-fold, binomial $P < 2.98 \times 10^{-13}$) binding sites (derived from ChIP-seq in IFN- α -stimulated K562 cells) within cis-eQTLs after IFN- β -stimulation.

Among the 57 genes (for example, *SLFN5*, *CLEC4F*, and *ARL5B*; Fig. 3F) with cis-reQTLs in all three stimuli, we observed that the most significant cis-reQTL in the (i) *SLFN5* locus (rs11080327) lies in an ENCODE ChIP-seq signal (29) for STAT1 (Fig. 4, A to C); (ii) *CLEC4F* locus (rs35856355) alters a commonly occurring cytosine in a canonical IFN-stimulated response element (ISRE) that is a target of IFN-activated TFs (Fig. 4, B and C) (22, 30–32); and (iii)

ARL5B locus (rs2130531) changes a guanine in the canonical ISRE motif (Fig. 4, B and C). Additional cis-reQTLs that alter putative STAT binding sites based on ChIP-seq data or predicted ISRE motifs include rs10086852 (*PTK2B*), rs1981760 (*NOD2*), rs73023464 (*C19ORF12*), rs1331717 (*IFI44*), and rs12064196 (*IFI44*) (Fig. 4A). Because STAT TFs are activated downstream of the type I IFN receptor (IFNAR) and bind to ISRE motifs (Fig. 4A) (22), we hypothesized that the SNPs in these sites are likely causal SNPs within their respective cis-reQTL regions.

Cis-reQTLs That Affect Differential Binding of Stimulus-Activated TFs

To experimentally validate our predicted regulatory mechanisms, we determined whether IFN- β -activated TFs bind differentially at these SNPs. Using radiolabeled 24- to 26-bp (base pair) double-stranded DNA (dsDNA) probes in electrophoretic mobility shift assays (EMSAs), we found

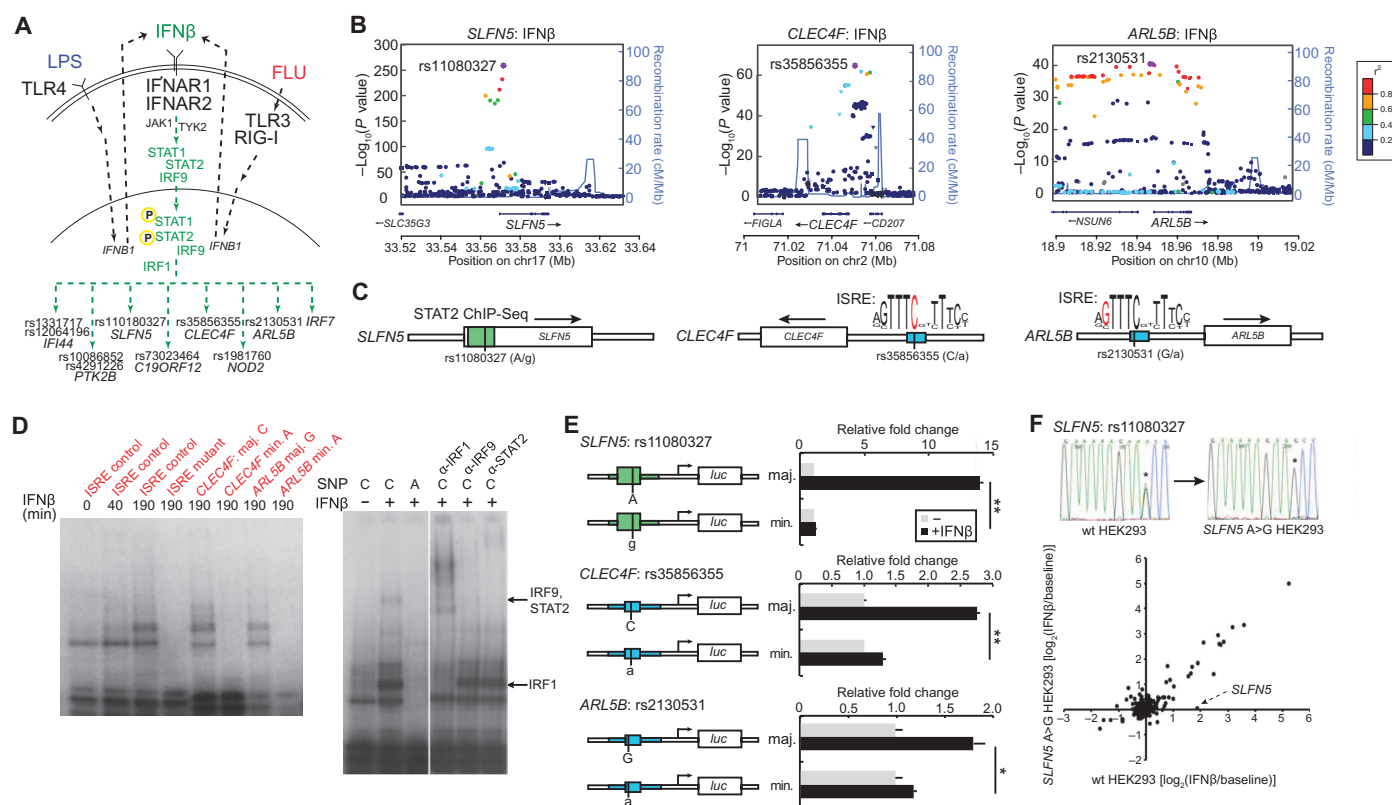


Fig. 4. Functional fine-mapping and mechanism of cis-reQTLs. (A) Pathway diagram of signal transduction cascade downstream of IFNAR activation. Activation of receptor leads to downstream activation of JAK-STAT cascade, leading to posttranslational activation of STAT and IRF TFs. (B) LocusZoom plots showing the $-\log_{10}(P)$ values of imputed cis-eQTLs (y axis) in the chromosomal regions (x axis) of *SLFN5*, *CLEC4F*, and *ARL5B*. The most significant imputed SNPs in each locus are labeled. (C) Schematic representation of alleles in the regions near the *SLFN5*, *CLEC4F*, and *ARL5B* genes that are in STAT2 ChIP-seq binding sites or that perturb ISRE motifs (SNPs are shown as vertical bars and in red letters). (D) EMSAs with 24- to 26-bp radiolabeled dsDNA probes—containing a known ISRE motif control, the *CLEC4F* rs35856355 major (C) or minor allele (A) sequences, or the *ARL5B* rs2130531 major (G) or minor allele (A) sequences—incubated with

nuclear lysates from IFN- β -stimulated MoDCs. On the right, supershift assays with or without antibodies against IRF1, IRF9, and STAT2 (designated α -IRF-1, and so on) with the *CLEC4F* rs35856355 major (C) probe are shown. (E) Luciferase expression from reporter constructs transfected into HEK293 cells that were left unstimulated or were stimulated with IFN- β (1000 U/ml) for 21 hours. Sequences (150 to 200 bp) from the major and minor haplotypes of the *SLFN5*, *CLEC4F*, and *ARL5B* regions were subcloned 5' of a minimal promoter and firefly luciferase gene. Firefly luciferase expression was normalized to *Renilla* luciferase expression expressed from cotransfected plasmid. (F) Fold change $\log_2(\text{IFN-}\beta \text{ stim/unstim})$ of signature genes in wild-type (wt) HEK293 cells (rs11080327^{wt}), plotted against fold change in CRISPR-converted (rs11080327^{ΔG}) HEK293 cells. Data are from one experiment representative of three [mean and SD shown in (E)]. * $P < 0.05$, ** $P < 0.01$, compared to unstimulated cells (Student's t test).

that probes encompassing the major alleles (rs35856355^C and rs2130531^G, which are associated with increased expression in *CLEC4F* and *ARL5B*), but not the minor alleles, shifted after incubation with IFN- β -stimulated MoDC nuclear lysates (Fig. 4D). Consistently, nonradiolabeled major, but not minor, allele probes competed for binding to the IFN- β -stimulated nuclear factors (fig. S4A). By incubating with antibodies to TFs known to bind the ISRE element, we found that IRF1 (IFN-regulatory factor 1) supershifted the low-molecular weight band and STAT2 and IRF9 supershifted the higher-molecular weight band, consistent with the known complex of these latter two proteins (32) (Fig. 4D). These results suggested that the SNPs in *CLEC4F* and *ARL5B* alter the binding of IFN-activated IRF1, STAT2, and IRF9 TFs.

To directly test the functional effects of the SNPs, we cloned 150- to 200-bp genomic regions surrounding rs11080327 (*SLFN5*), rs35856355 (*CLEC4F*), and rs2130531 (*ARL5B*) upstream of

a luciferase reporter driven by a minimal promoter (Fig. 4E). For each region, we created two constructs that differ only at the respective SNPs. Because almost all cell types respond to IFN- β stimulation, we transfected the reporter constructs into human embryonic kidney 293 (HEK293) cells and stimulated the cells with IFN- β . Consistent with our hypothesis, the constructs containing the major alleles of rs11080327 (*SLFN5*), rs35856355 (*CLEC4F*), and rs2130531 (*ARL5B*) induced 11.6-, 2.1-, and 1.5-fold, respectively, more luciferase production than the cognate constructs containing the minor alleles (*t* test, $P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively) (Fig. 4E). Furthermore, mutation of motif-conserving nucleotides across the *CLEC4F* ISRE motif reduced induction by IFN- β , whereas mutation of nonconserved nucleotides did not (fig. S4B), consistent with the ISRE consensus motif.

Finally, to further test (33) the functionality of rs11080327—the *SLFN5* variant with the strongest functional effect—in its native chromo-

some, we directly edited the genome using the CRISPR system (34, 35), converting the heterozygous rs11080327^{A/G} in HEK293 cells to a homozygous rs11080327^{G/G} (Fig. 4F and fig. S4C). We then stimulated both native and CRISPR-converted cells with IFN- β . The fold induction of *SLFN5* decreased from 3.64 in heterozygous cells to 1.03 in the converted rs11080327 (G) homozygotes, whereas the induction of other genes was unaffected (Fig. 4F and fig. S4C, independent CRISPR construct).

The enrichment, EMSA, reporter assays, and directed mutagenesis results suggest that we have identified causal variants in some of these cis-eQTL regions and demonstrate that the reQTLs occur because of differential binding of stimulus-activated TFs.

A Trans-eQTL That Also Associates with IRF7 as a Cis-eQTL

We next attempted to detect cases where variation in gene expression is explained by distant

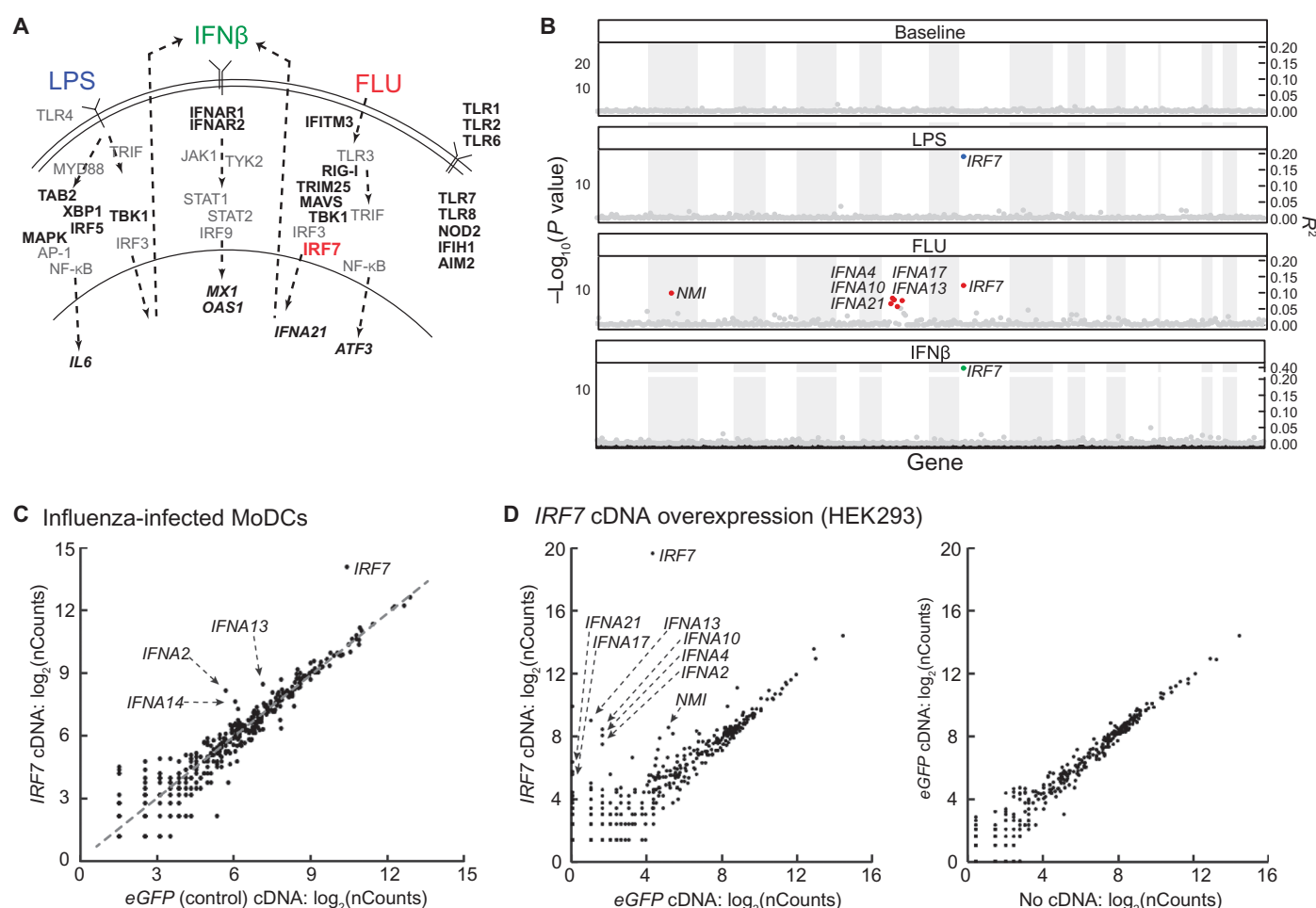


Fig. 5. Trans-reQTL association at the *IRF7* cis-regulatory locus. (A) Diagram showing selected components of TLR4, TLR3, RIG-I, and IFNAR pathways. Components with significant cis-eQTLs (permutation FDR < 0.05) are shown in black (or red if they also have a trans-eQTL); components that do not have significant cis-eQTLs are gray. (B) Manhattan plot showing the trans-association of rs12805435 to all 415 genes on signature gene set in baseline, LPS-stimulated, FLU-infected, and IFN- β -stimulated conditions. Trans-eQTL

to *NMI* and cluster of *IFN- α* genes (*IFNA4*, *IFNA10*, *IFNA13*, *IFNA17*, and *IFNA21*) are labeled. (C) Expression [log₂(nCounts)] of 415 signature genes in FLU-infected MoDCs overexpressing *IRF7*, plotted against expression in FLU-infected MoDCs overexpressing *eGFP* control. (D) Expression [log₂(nCounts)] of 415 signature genes in HEK293 cells overexpressing *IRF7*, plotted against expression in HEK293 cells overexpressing *eGFP* control. Right, expression of genes in cells with *eGFP* overexpression versus cells without cDNA.

genetic variants acting in trans. We noted that cis-eQTLs for regulator genes tend to have smaller effect sizes and less significant P values than those for regulated genes (fig. S3E). To decrease the multiple testing burden of detecting trans-associations, we restricted testing SNPs local to genes on our gene signature set. A number of these are cis-eQTLs associated with the expression of genes encoding regulators (for example, TFs) in pathways that we stimulated (Fig. 5A). Of these, rs12805435 was the most significant, associating with the expression of the master antiviral TF *IRF7* in cis only after influenza, LPS, or IFN- β stimulation (table S4, A to D). This SNP associated with both the expression and induction of seven additional genes in trans (that is, the transcriptional start site or stop codon is located >1 Mb from the SNP) only after influenza infection: *NMI*, *IFNA4*, *IFNA10*, *IFNA13*, *IFNA21*, *IFNA17*, and *IFNA5* ($P < 6.26 \times 10^{-5}$ in expression, $P < 1.68 \times 10^{-5}$ in differential expression) (Fig. 5B and tables S8 to 10).

To test whether the trans-associated genes are indeed targets of IRF7, we infected MoDCs

that overexpressed *IRF7* or a control gene with influenza virus, and found that *IRF7* overexpression induced the expression of *IFNA2*, *IFNA13*, and *IFNA14* in influenza-infected cells (Fig. 5C). In addition, in HEK293 cells, which normally express very low levels of *IRF7*, we overexpressed *IRF7* and observed induction of *NMI*, *IFNA4*, *IFNA10*, *IFNA13*, *IFNA14*, and *IFNA21* (Fig. 5D), suggesting that IRF7 is sufficient to drive downstream expression. We have thus identified a stimulus-specific cis-eQTL associated with *IRF7* expression, which is also a trans-reQTL that underlies the variability of IFN induction in response to influenza infection in humans.

DC reQTLs That Overlap with Autoimmune and Infectious Disease SNPs from GWAS

We examined the subset of loci associated in GWAS with inflammatory disorders. We first extended an analysis of Crohn's disease in which loci are enriched for genes specifically expressed in DCs (12, 14) to multiple sclerosis, celiac disease, psoriasis, and leprosy (fig. S5A). We found that genes nearest to the susceptibility loci for

these diseases were enriched not only in DC-specific genes but also in genes induced by LPS and/or influenza (fig. S5B), which suggests that some of the GWAS loci modulate the expression of the corresponding genes in activated DCs.

Supporting this hypothesis, 15 cis-reQTLs and 23 cis-eQTLs that were not significant at baseline but only became significant after stimulation are the same SNPs previously identified in GWAS of autoimmune and infectious diseases, including Crohn's disease, multiple sclerosis, celiac disease, psoriasis, and leprosy (25) (Fig. 6, A and B, and table S11). These include *NOD2* with leprosy (rs9302752), *IRF7* with systemic lupus erythematosus (SLE) (rs4963128), *TRAF1* with rheumatoid arthritis (rs881375), and *CREM* with Crohn's disease (rs12242110) and ulcerative colitis (rs4246905). For example, rs9302752—a SNP previously associated with susceptibility to leprosy (17)—was associated in our study with the absolute and fold expression of *NOD2* under IFN- β stimulation ($P = 3.49 \times 10^{-25}$) but not at baseline (Fig. 6A and table S11); *NOD2* plays a known role in pathogen sensing and possibly mycobacterial

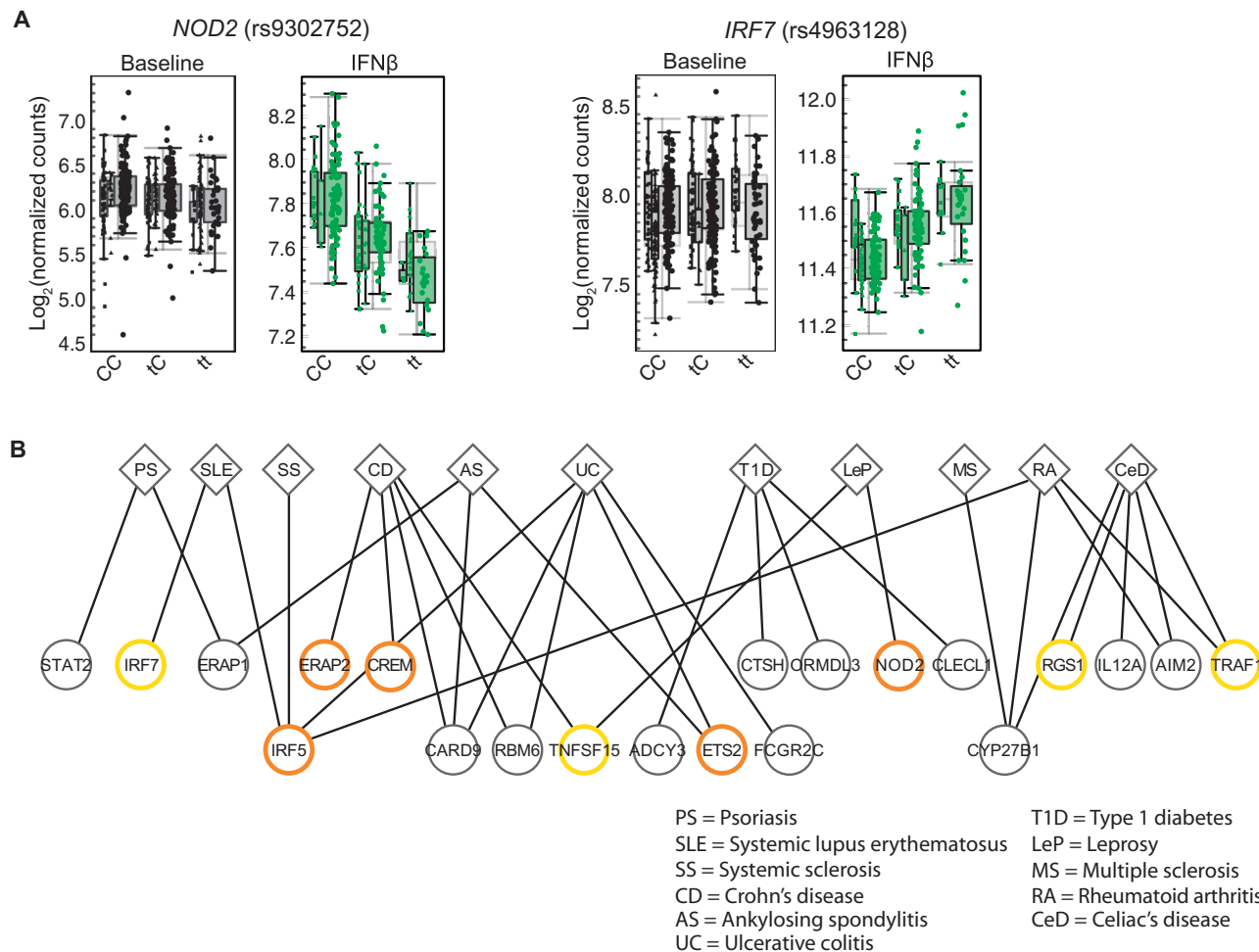


Fig. 6. Autoimmune and infectious disease-associated SNPs are cis-eQTLs and cis-reQTLs. (A) Expression [$\log_2(\text{nCounts})$] of *NOD2* in resting and IFN- β -stimulated MoDCs from 184 Caucasians as a function of genotype of the leprosy GWAS SNP, rs9302752 (left). (Right) expression [$\log_2(\text{nCounts})$] of *IRF7* in resting and IFN- β -stimulated MoDCs from 184 Caucasians as a

function of genotype of the SLE GWAS SNP, rs4963128. (B) Plot showing overlap of genome-wide significant ($P < 5 \times 10^{-8}$) GWAS SNPs with cis-eQTLs and reQTLs in MoDCs, with clinical phenotypes connected to corresponding gene expression phenotypes by lines. Orange circles represent cis-reQTLs ($P < 10^{-7}$); yellow circles represent stimulus-specific cis-eQTLs ($P < 10^{-7}$).

immunity (36). Similarly, rs4963128—a variant associated with SLE (37)—was associated in our study with the expression of *IRF7* after IFN- β stimulation ($P = 1.10 \times 10^{-16}$) but not at baseline (Fig. 6A), in line with the importance of type I IFN responses in SLE pathogenesis (38). We note that rs4963128 is on the same haplotype ($R^2 = 0.69$, $D' = 0.94$) as the *IRF7* SNP rs12805435 that is associated with the trans-reQTL effect described above. These results suggest a role for innate immune pathogen-sensing pathways in the pathogenesis of these inflammatory disorders.

Discussion

Although genetic association studies have identified alleles that confer disease risk, little is known about how these genetic variants contribute to disease through their effects on specific biological processes and their interaction with environmental stimuli. We addressed this question by quantifying the DC response to pathogens in a set of genotyped individuals, and then leveraged our understanding of these pathways to explain the mechanisms underlying the observed genotype-environment-phenotype interactions.

Many of the QTL associations we identified are only detectable in the presence of specific stimuli, which underscores the need to activate cells to capture additional genotype-phenotype relations (39–41). Furthermore, few eQTL studies have definitively identified the causal variants underlying the associations. By measuring variability in hundreds of individuals, applying stimuli that partially overlap in their downstream pathways, and leveraging genomic data sets such as the 1000 Genomes Project and ENCODE (29, 42), we (i) pinpointed causal variants in reQTL regions and (ii) identified the stimulus-activated TFs that bind differentially at these SNPs, which explain some of the $G \times E$ interactions. Our data set can thus be used to explore mechanisms of $G \times E$ interactions (43) and are consistent with deoxyribonuclease I sensitivity QTL (44) and ChIP-seq QTL (45) studies that showed that differential TF binding between individuals is pervasive in resting cells.

The reQTLs we identified provide genetic explanations for interindividual variation in innate immune responses. This is best exemplified by the trans-reQTL in the *IRF7* locus that regulates the type I IFN antiviral response. Our study reveals the effects of this trans-reQTL on target genes (antiviral IFN module) in the context of a particular cell type (DCs) and in response to specific ligands (influenza). The changes in this immune response are, in turn, likely to affect organismal phenotypes that are driven by the IFN module, including susceptibility to viral infections and autoimmune diseases like SLE.

Overall, our high-throughput experimental pipeline and integrative analysis of primary human DCs reveals abundant gene-by-environment interactions, points to the effects of disease variants on pathogen detection, and motivates

extending our approach to a wide variety of immune cell types and stimulatory conditions to better explain the impact of human genetic variation on the immune response.

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Supplementary Materials

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Quantifying Molecular Stiffness and Interaction with Lateral Force Microscopy

Alfred John Weymouth,* Thomas Hofmann, Franz J. Giessibl

The spatial resolution of atomic force microscopy (AFM) can be drastically increased by terminating the tip with a single carbon monoxide (CO) molecule. However, the CO molecule is not stiff, and lateral forces, such as those around the sides of molecules, distort images. This issue begs a larger question of how AFM can probe structures that are laterally weak. Lateral force microscopy (LFM) can probe lateral stiffnesses that are not accessible to normal-force AFM, resulting in higher spatial resolution. With LFM, we determined the torsional spring constant of a CO-terminated tip molecule to be 0.24 newtons per meter. This value is less than that of a surface molecule and an example of a system whose stiffness is a product not only of bonding partners but also local environment.

True atomic resolution was demonstrated for atomic force microscopy (AFM) more than 15 years ago, but only recently has it been possible to atomically engineer the tip apex (1–4), a technique pioneered in the scanning tunneling microscopy community (5, 6). Tip termination with a carbon monoxide (CO) molecule has made studies of inter- and intramolecular bonding possible (7–11). The CO-terminated tip itself, however, has proven to be difficult to characterize, despite experimental and theoretical approaches (12, 13), in part because it is challenging to characterize lateral forces and stiffnesses with AFM. With any study of short-range interactions, AFM requires that the long-range background interaction of the macroscopic tip and surface be subtracted (14). Although a full three-dimensional (3D) data set can reconstruct the potential energy, lateral forces and stiffnesses are only accessible by taking a numerical derivative of experimentally determined energies (15), which results in substantial noise in the data.

In lateral force microscopy (LFM), the tip is oscillated parallel to the surface (16, 17), and the recorded frequency shift (Δf) is a direct measure of the lateral stiffness (18). The subtraction of long-range contributions—which is necessary in normal-force AFM (in which the tip oscillates normal to the surface) in order to identify the short-range force components—is obsolete in LFM, rendering LFM particularly appealing for measurements in which the short-range interaction is the signal of interest (19). We have used both normal-force AFM and LFM to characterize a CO-terminated tip and quantify parameters of a model incorporating torsional springs to account for the response of the CO molecules to lateral forces.

When the CO tip was first used to image pentacene, it was remarked that the image seemed to be distorted because of CO relaxation (7). Further experiments showed that the relaxation of

the CO molecule on the tip was an important part of the tip-surface interaction (13). This relaxation is usually characterized as a torsional spring, with the CO bending around the metal atom to which it is bound (12).

We used a CO-terminated tip to probe a single CO molecule on Cu(111). CO on Cu(111) has been well-studied, and the system offers a high degree of symmetry. The asymmetry in a normal-force AFM image of a CO with a CO-terminated tip (Fig. 1A) is the result of the slight asymmetry of the tip with respect to the surface, amplified by the bending of both tip and surface CO molecules. Images collected at further distances [such as those shown in fig. S1 (20)] do not show this degree of asymmetry.

We acquired a 3D data set by collecting constant-height, normal-force AFM images at various distances from the surface (21). To isolate the short-range interaction, we subtracted

the raw Δf data from the Δf signal above the bare copper surface (14). The data were then deconvoluted and integrated twice along the z direction (where z denotes the distance to the surface) to yield the potential energy (21–23). The potential energy map of the short-range interaction at closest approach is shown in Fig. 1B.

A plot of the energy as a function of tip height above the surface CO molecule is given in Fig. 1C. Also included in Fig. 1C is a fit of the energy-distance curve to a Morse potential (24)

$$E_M = E_B \left(-2 \exp \left[-\frac{r - \sigma}{\lambda} \right] + \exp \left[-2 \frac{r - \sigma}{\lambda} \right] \right) \quad (1)$$

The distance r describes the core-core distance between the two oxygen atoms. We comment on the equilibrium distance, σ , later. The bond energy, $E_B = 8.4$ meV, was determined by the energy minimum, and the decay length, $\lambda = 47$ pm, was determined with a least-squares fit from the data further out from the equilibrium distance. For these longer distances, the interaction is attractive, and the molecules do not bend. At closer distances, we expect the repulsive interaction to lead to the CO molecules bending away from each other.

We then used a lateral force sensor (17)—also with a CO-terminated tip—to probe a surface CO molecule. The mathematical description of the frequency shift and its relation to force remains the same: Interaction with the surface changes the frequency of oscillation proportional to the force gradient, k_{ts} , along the direction of oscillation. The effect of the cantilever oscillation is that we measured a weighted average, $\langle k_{ts} \rangle$, over the cantilever's oscillation (25). The frequency shift was directly proportional to $\langle k_{ts} \rangle$, via the spring constant of the cantilever, k , and its resonance frequency f_0 : $\Delta f = \langle k_{ts} \rangle f_0 / (2k)$.

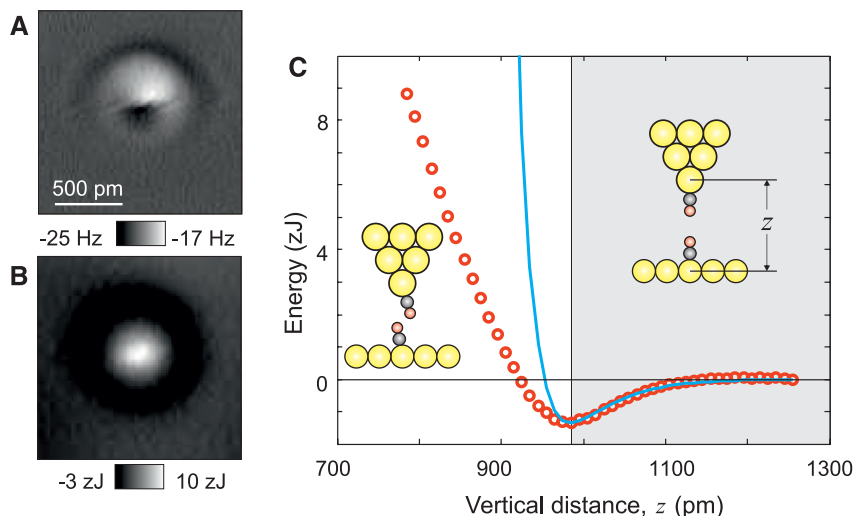


Fig. 1. Normal-force AFM data with a CO-terminated tip. (A) Normal AFM data of a CO molecule with a CO-terminated tip. (B) Deconvoluted energy image. (C) Energy as a function of vertical tip position when the tip is directly above the CO molecule. Red dots are data, and the blue curve is a fit of a Morse potential to the data.

Universität Regensburg, Regensburg 93053, Germany.

*Corresponding author. E-mail: jay.weymouth@ur.de

The raw image of the CO molecule with LFM (Fig. 2A) revealed a circular shape with a highly localized depression in the middle. The high spatial resolution in this LFM image could not be reconstructed from data acquired with a normal-force AFM: Similar to the method used in (15), we can evaluate lateral stiffnesses via the potential energy from the normal-force AFM data (Fig. 2A, inset), but after the two numerical derivatives, the sharp localized depression is not reproduced. Although one solution to the noise caused by the derivatives would be to use a low-pass filter, this step would only decrease the spatial resolution. Thus, it was not feasible to reconstruct this sharp feature by using only normal-force AFM data.

We next acquired constant-height LFM slices above the CO molecule. A line scan through the image is shown in Fig. 2C at closest approach over the CO molecule in the direction of the tip oscillation. Because this line was in the same direction as the tip oscillation, it could be deconvoluted and integrated so as to yield lateral force in that direction. We repeated this process for several tip-sample distances. The lateral force over the CO molecule is depicted in the colored graph in Fig. 2D as a function of both lateral and vertical position. As we expect from the normal-force AFM data, the lateral force shows attractive interaction as the CO molecules approach each other, then repulsive interaction closer than the equilibrium distance. Assuming that the CO bends to relax, we would expect CO bending for all positions except when the lateral forces are equal to zero.

We calculated the lateral and vertical position where the lateral forces are equal to zero. These zero crossings were fit to a circle, as shown in Fig. 2D. This result implies that there was a very weak angular dependence for the CO-CO interaction,

which is another advantage of a CO-terminated tip. The zero crossings of the force are the locations of the energy minimum, and thus, the radius of the circle, 385 pm, is the equilibrium distance between the oxygen atoms, σ . Absolute height determination is also a challenge in scanning-probe techniques. With the assumption that the interaction is not directionally dependent, σ can be used to determine the absolute height of our tip above the surface CO molecule, as given in Fig. 2D.

We next attempted to determine the relaxation of the CO molecule experimentally. In order to make this problem tractable, we separated the total interaction into three components: (i) the interatomic interaction between the two oxygen atoms, (ii) the CO relaxation of the tip molecule, and (iii) the CO relaxation of the surface molecule. We propose the following model: (i) The interatomic interaction can be described by a Morse potential, which we have fully characterized with E_B , λ , and σ . (ii) The surface CO molecule responds as a torsional spring, with a torsional spring constant of $\kappa_S = 150$ zepto-Newton meter (zNm) and a moment arm of $l_{CO} = 302$ pm (26). (iii) The tip CO molecule relaxes as a torsional spring with an unknown spring constant κ_T but with the same moment arm l_{CO} . The moment arm was determined by a theoretical study that determined the core-core distance of the copper surface atom to the carbon and oxygen atoms (26). Helium scattering measurements have previously determined the energy of the frustrated translational mode to be 4 meV (27). With the moment arm, these values can then be used to determine κ_S as described in (3).

A schematic of the distances in the model is shown in Fig. 3A. At every position of the final

tip atom that does not relax (x, z), the total energy of the system can be calculated as a function of the bending angle of the tip CO molecule θ_T and of the surface CO molecule θ_S

$$E = E_M(r) + \frac{1}{2} \kappa_T \theta_T^2 + \frac{1}{2} \kappa_S \theta_S^2 \quad (2)$$

where r is the core-core distance between the oxygen atoms, which is a function of x, z, θ_T , and θ_S . For every tip position (x, z), we minimize the energy with respect to θ_T and θ_S , which thus yields the energy of the system at that position. We can differentiate this energy surface to yield the force and force gradient. The force gradient can then be convoluted with a semicircular weight function (to account for the cantilever motion) and multiplied by $f_0/(2k)$ to explicitly determine Δf , which can be compared directly with our data.

A spectrum of LFM data in red points (Fig. 3B) was collected as a function of the vertical distance of the tip above the CO molecule. For a given value of κ_T , we can use the model to generate an equivalent spectrum. We varied κ_T for a least-squares fit to the data. The best fit, shown by a blue solid line in Fig. 3B, yielded a value of $\kappa_T = 22$ zNm. This value can be expressed as a linear spring constant $k_T = \kappa_T/(l_{CO})^2 = 0.24$ N/m. We can then simulate a full LFM image from this model (Fig. 2B). The agreement is easier to appreciate comparing line scans (Fig. 2C). Data and model output further from the surface (fig. S2) (20) also show excellent agreement.

Previous density functional theory-based calculations of k_T ranged from 0.3 to 1.6 N/m, with previous experimental evidence pointing to a value lower than 0.5 N/m (12). Our value of 0.24 N/m agrees with this assessment, in particular

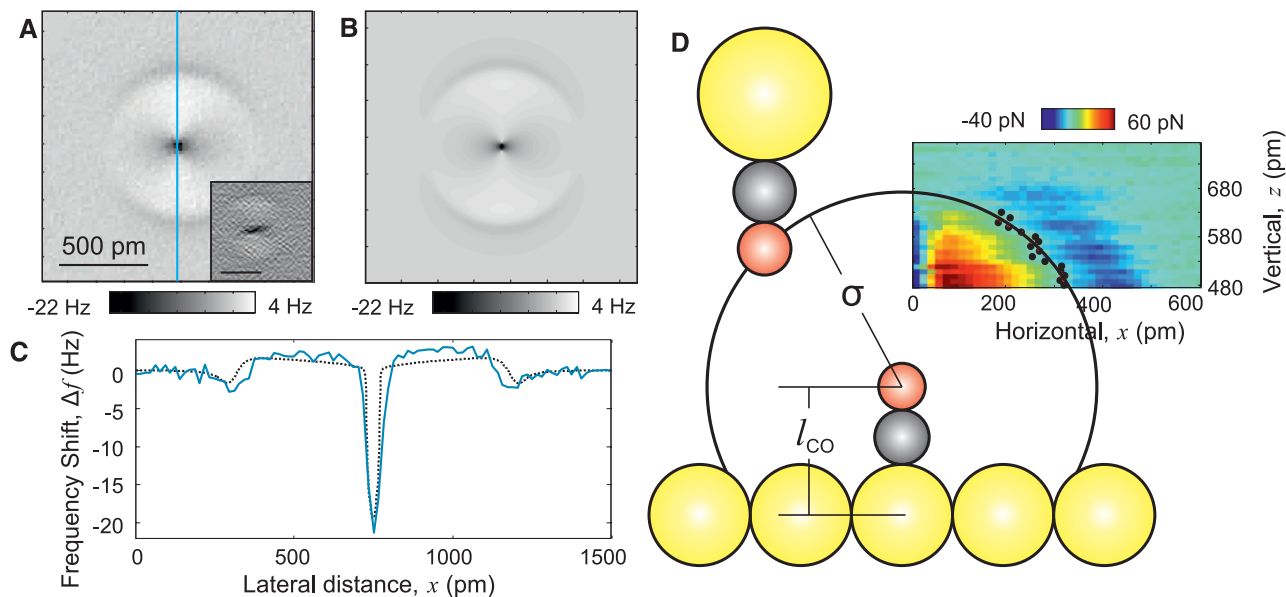


Fig. 2. LFM with a CO-terminated tip. (A) Raw data from a LFM sensor. Tip oscillation is in the same direction as the solid blue line. (Inset) Lateral stiffness as evaluated from normal AFM data. Scale bar, 500 pm. (B) Model output with the same scale as (A). (C) A line scan through the center of the

CO molecule in the direction of oscillation. Solid blue line is the data; dashed black line is model output. (D) The colored inset shows the lateral forces. Black points indicate where the lateral force is zero. These points lie on a circle whose radius defines the bond length between the oxygen atoms.

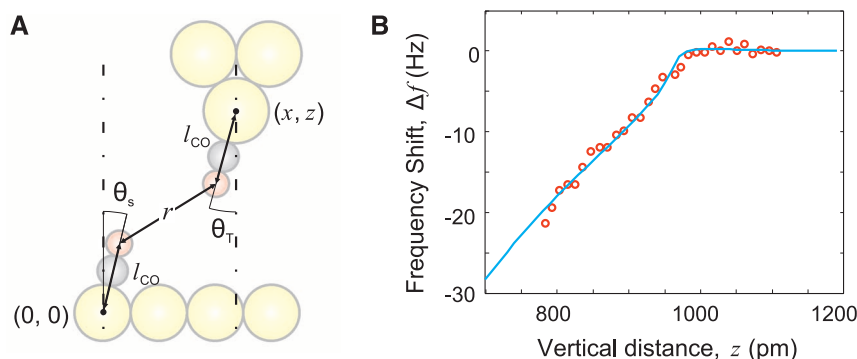


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The LFM images have a strong signal that is much more spatially confined than are the normal-force AFM images. Physically, the spatial resolution of frequency-modulation AFM is limited by the lateral extent of the force gradient. Especially in the case of a CO-terminated tip—in which lateral forces change rapidly over a sharp transition,

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Simultaneous Ground- and Space-Based Observations of the Plasmaspheric Plume and Reconnection

B. M. Walsh,^{1,2*} J. C. Foster,³ P. J. Erickson,³ D. G. Sibeck¹

Magnetic reconnection is the primary process through which energy couples from the solar wind into Earth's magnetosphere and ionosphere. Conditions both in the incident solar wind and in the magnetosphere are important in determining the efficiency of this energy transfer. In particular, the cold, dense plasmaspheric plume can substantially impact the coupling in the dayside reconnection region. Using ground-based total electron content (TEC) maps and measurements from the THEMIS spacecraft, we investigated simultaneous ionosphere and magnetosphere observations of the plasmaspheric plume and its involvement in an unsteady magnetic reconnection process. The observations show the full circulation pattern of the plasmaspheric plume and validate the connection between signatures of variability in the dense plume and reconnection at the magnetopause as measured in situ and through TEC measurements in the ionosphere.

The upward extension of cold, dense ionospheric plasma forms Earth's plasmasphere. The motion of the plasmaspheric particles is governed by two electric fields, resulting from corotation and convection motion. The first dominates close to Earth and enforces rotation of these particles with Earth. The second comes from solar

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region of the plasmasphere into a drainage plume that extends sunward toward the dayside magnetopause (*1, 2*) and noontime cusp ionosphere (*3*).

The spatial and temporal evolution of the plasmasphere-ionosphere plume can be measured through observations of total electron content (TEC) from ground-based Global Positioning Satellite (GPS) receivers. Integrated electron content is obtained through monitoring the phase delay of radio signals received from GPS spacecraft in circular orbits at an altitude of 20,000 km. These ground-based observations allow us to monitor the large-scale morphology and extent of the plasmaspheric plume (*4*). The structure and development of a sunward-extending plume in the dusk sector, as seen in TEC measurements, has been confirmed through extreme ultraviolet (EUV) imaging with NASA's IMAGE spacecraft (*3*); however, the sensitivity of these measurements does not allow for detection of plumes with lower densities and with large radial distances close to the magnetopause. Although the extension of the plume to the outer

¹NASA Goddard Space Flight Center, Greenbelt, MD 20771, USA. ²Space Sciences Laboratory, University of California, Berkeley, CA 94720, USA. ³MIT Haystack Observatory, Westford, MA 01886, USA.

*Corresponding author. E-mail: brian.walsh@nasa.gov



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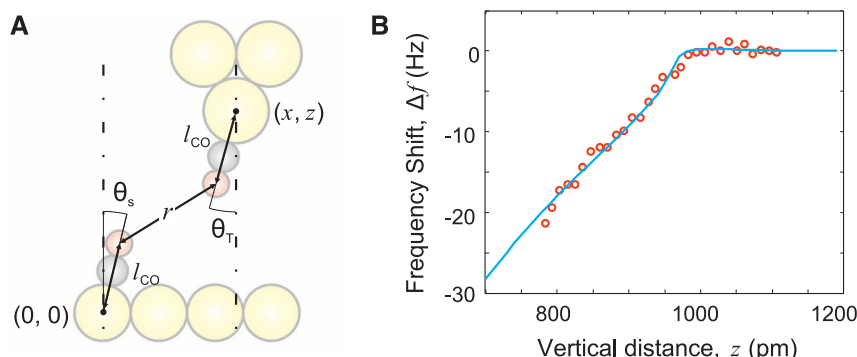


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*Corresponding author. E-mail: brian.walsh@nasa.gov

magnetosphere has been inferred from ground-based and in situ measurements independently, coincident in situ spacecraft and ground-based measurements necessary to confirm the plume's full extension to the magnetopause and to the magnetic reconnection point have not been presented.

Fig. 1. GPS TEC maps showing temporal evolution of the SED plume at noontime cusp. The color scale shows TEC units (TECU) where 1 TECU = 10^{16} electrons/m². The plots are oriented so that local noon is up in each panel. The plume persists for a number of hours; the black arrow indicates the position of the cusp signature in the TEC data. The gray circle in the top left panel is the magnetic footprint of THEMIS E as it crosses the reconnecting magnetopause at 18:22 UT. The black circle is the poleward precipitation boundary from the OVATION model (21).

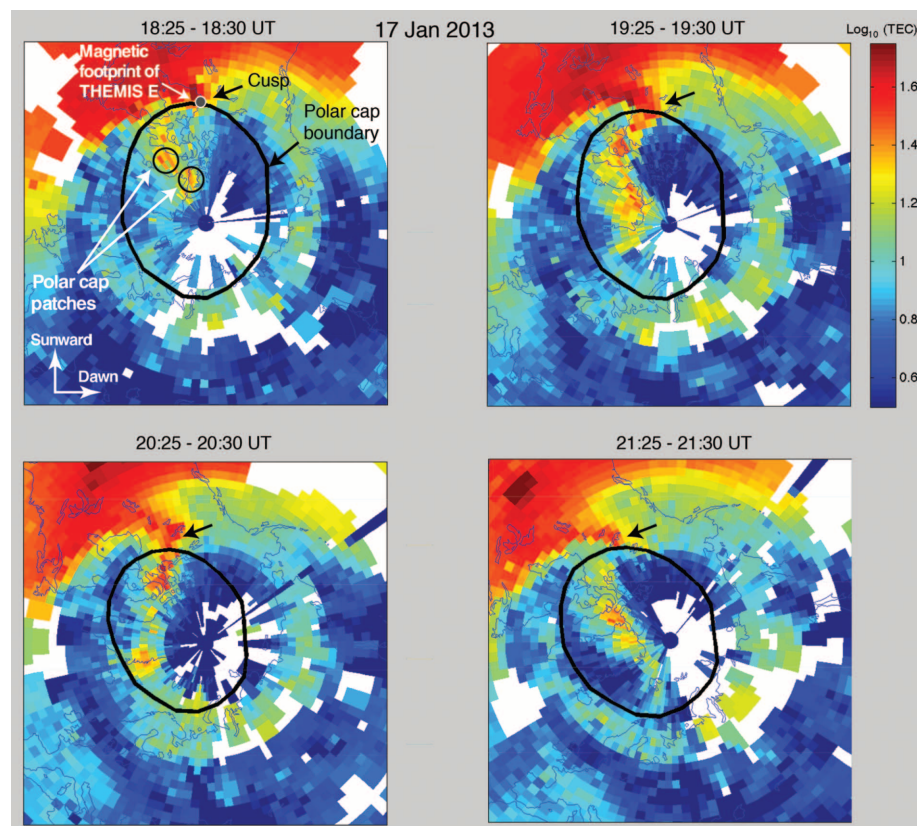


Fig. 2. TEC measurements projected to the equatorial plane following magnetic field lines with the International Geomagnetic Reference Field (IGRF) model. The star indicates the position where the THEMIS A spacecraft crosses the magnetopause. The solid circles along the orbit are each separated by 1 hour spanning the time period from 16 to 22 UT. The black line is a modeled position of the magnetopause (22).

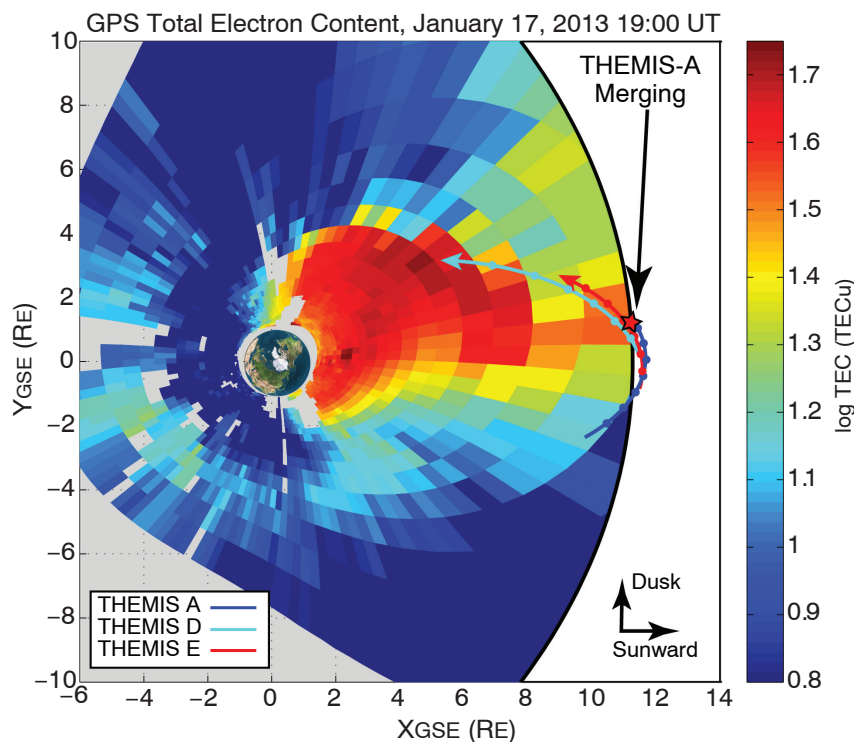


Fig. 3. Measurements from the three THEMIS spacecraft during each magnetopause crossing.

The left plots show the same time scale for each spacecraft in GSM coordinates. The right plots are zoomed in on the time period of the magnetopause crossing and are in boundary normal (LMN) coordinates. For each spacecraft, the panels from top to bottom show electron density, bulk flow components, and magnetic field components. The LMN coordinate system is defined such that the N axis points outward along the magnetopause normal and the (L, M) plane is tangential to the magnetopause with L orientated due north and M due west. The vertical dashed black line indicates when each spacecraft passed through the magnetopause.

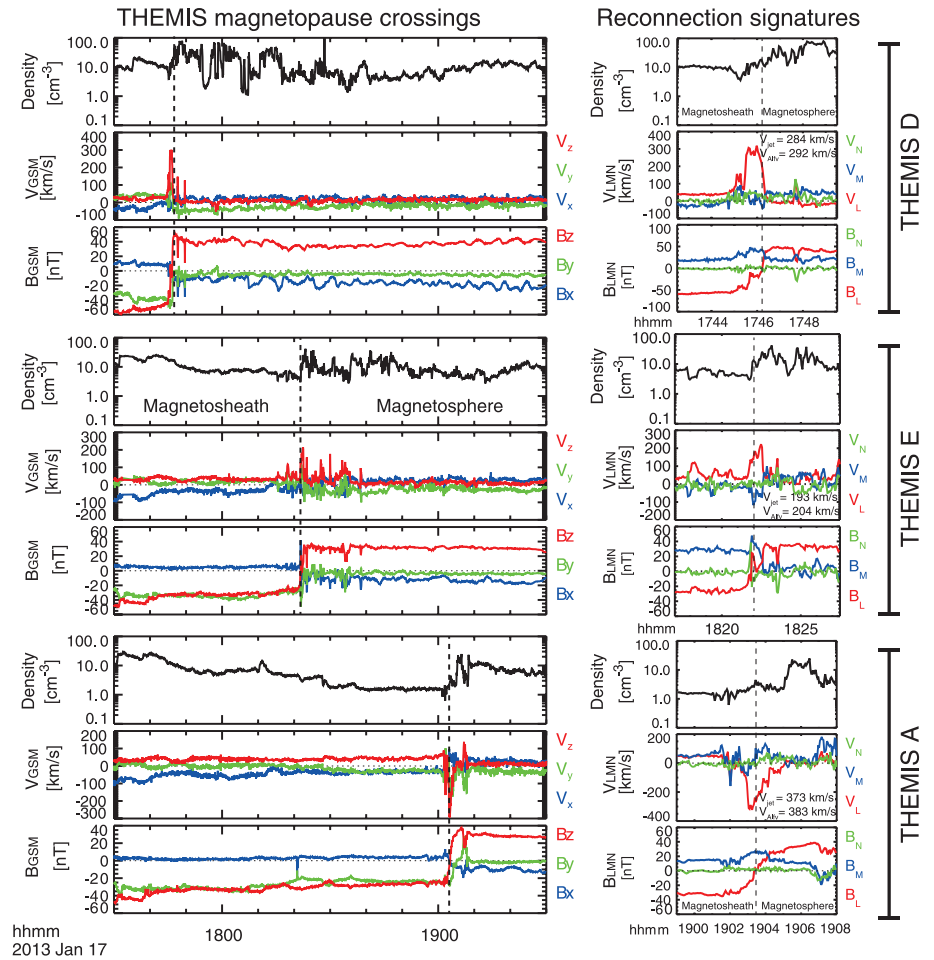
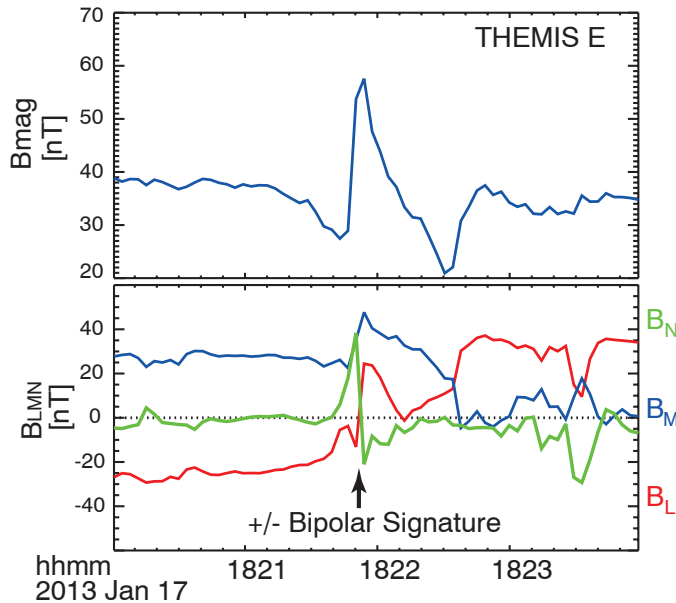


Fig. 4. Magnetic field measurements from THEMIS E during the magnetopause crossing in boundary normal coordinates.

Clear signatures of a flux transfer event were observed. There is an enhancement in the magnetic field strength (top panel) corresponding to a bipolar signature in the B_N component and a deflection of the B_M component (bottom panel). Because the spacecraft is north of the reconnection site, as noted from the jets in the $+V_L$ direction (Fig. 3), an outward/inward (+, -) bipolar signature, indicating a northward-moving feature, is anticipated (23) and observed.



when the plasma density increases (9, 10). Here we present simultaneous, magnetically coincident observations of the plume at the dayside reconnection point in both the magnetosphere and ionosphere.

On 17 January 2013, a coronal mass ejection (CME) impacted Earth's magnetosphere, resulting in a small geomagnetic storm, as indicated by the Disturbed Storm Time (*Dst*) index (minimum of

−53 nT). During the storm, the ionospheric density increased and a long-duration storm-enhanced density (SED) plume (*I*) developed in TEC measurements (Fig. 1). The SED plume streamed in the convection flow from the dusk sector to the noon-time cusp (arrow) and then antisunward across the polar cap, forming the polar tongue of ionization (TOI) (12). The patchy, intermittent structure of enhanced TEC within the TOI (Fig. 1) has been inferred to indicate variability in the rate of magnetopause reconnection (13, 14).

During the same time period when the extended SED plume was monitored in the ionosphere, three of NASA's THEMIS spacecraft (15) detected the plume with in situ measurements at the magnetopause in a "string of pearls" formation (Fig. 2). The local time of the plume projected from the ionosphere is consistent with the location where THEMIS observed high-density plasma at the magnetopause (12.7 hours in magnetic local time), demonstrating the continuous extension of the plume from the inner magnetosphere sunward all the way to the magnetopause.

The THEMIS spacecraft were separated by roughly 45 min along the orbit. During the time plotted on the left in Fig. 3, each spacecraft passed from the magnetosheath into the magnetosphere. The magnetopause boundary is identified through a strong rotation in the *Z* magnetic field compo-

nent from -45 nT to 40 nT in the geocentric solar magnetospheric (GSM) coordinate system. This coordinate system is defined such that the X axis points from Earth to the Sun, Y is perpendicular to Earth's magnetic dipole axis so that the XZ plane contains the dipole axis, and Z is in the same sense as the northern magnetic pole. For each magnetopause crossing, the magnetospheric density adjacent to the magnetopause was greater than 10 cm^{-3} . This is close to two orders of magnitude greater than the nominal value, demonstrating the presence of a plume. The density was measured through the spacecraft potential.

During each of the three magnetopause crossings, a strong rotation of the magnetic field ($>120^\circ$) and a reconnection jet were observed. These crossings provide several confirmations of magnetic reconnection and the participation of the dense plume in the process. The first confirmation is the jet velocity. The reconnection jet is caused by the magnetic tension force that accelerates the exhaust plasma to the Alfvén speed. In the case of asymmetric reconnection, when the density and magnetic field strength are not the same on both sides of the current sheet, the jet velocity is a hybrid of Alfvén speeds on both sides. In each boundary crossing, the jet velocity matches to within 5% of the predicted hybrid Alfvén speed (Fig. 3) as calculated by a previous model (9, 16).

A second confirmation of the impact of the cold plume plasma is the location of the reconnection jet. Typically the magnetosheath is much denser than the dayside magnetosphere and the jet lies primarily on magnetic field lines with magnetospheric orientation ($+B_L$) (17, 18). In our study, the region of the magnetosphere adjacent to the magnetopause has been mass-loaded and the jets are primarily on field lines with magnetosheath orientation ($-B_L$) (Fig. 3). The occurrence of reconnection jets primarily on field lines of magnetosheath orientation provides additional evidence for the impact of the plume density on magnetopause reconnection.

The location of THEMIS at the reconnecting magnetopause also maps to the point in the ionosphere where the TOI is formed and enhancements in TEC stream tailward over the pole on open field lines (Fig. 1). This confirms that the formation of the TOI in the ionosphere is spatially linked to the presence of the plume and reconnection at the magnetopause. The dense plasma on newly opened magnetic field lines convects tailward over the pole, as observed in the motion of TOI patches in the ionosphere and in situ at the magnetopause. Future studies using the high spatial and almost continuous coverage of the ground-based TEC maps may use this connection to monitor reconnection and identify when plume material has reached the magnetopause.

In addition to identifying substantial density enhancements and mass loading at the magnetopause, these conjugate measurements confirm the connection between intermittent reconnection signatures at the magnetopause and in the ionosphere. Bursts of reconnection can cause twisted

magnetic flux ropes known as flux transfer events to form at the magnetopause (19, 20). At the magnetopause, a flux transfer event, demonstrating intermittent or “bursty” reconnection, was observed in the THEMIS measurements (Fig. 4). In the ionosphere, patches of TEC enhancements known as polar cap patches were observed convecting over the pole on open magnetic field lines (Fig. 1). These patches correspond to variability in reconnection causing uneven rates of plasma to be transported over the pole. Previous work (13, 14) used polar cap patches to infer variability in magnetopause reconnection; however, this association had not been validated through in situ measurements at the magnetopause.

Our results imply an extended plasmaspheric plume that spanned from a nominal plasmopause to the dayside magnetopause, where it impacted magnetic reconnection. Ground-based TEC measurements demonstrate that the extended plume existed for several hours. During this period, three THEMIS spacecraft provided magnetically coincident measurements of the plume mass-loading the magnetopause reconnection site near local noon. The spacecraft measurements show signatures of intermittent or bursty reconnection at the magnetopause corresponding to the occurrence of patches of enhanced TEC convecting tailward on open field lines over the pole in the ionosphere. The simultaneous observation of the conjugate location of the reconnection site at the magnetopause with the TEC cusp signature ionosphere (Fig. 1) is important. With this association validated, studies of the occurrence, location, and variability of reconnection at the magnetopause can be conducted with the assistance of ground-based TEC maps that provide high spatial and almost continuous temporal coverage.

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Tunable Phonon Polaritons in Atomically Thin van der Waals Crystals of Boron Nitride

S. Dai,¹ Z. Fei,¹ Q. Ma,² A. S. Rodin,³ M. Wagner,¹ A. S. McLeod,¹ M. K. Liu,¹ W. Gannett,^{4,5} W. Regan,^{4,5} K. Watanabe,⁶ T. Taniguchi,⁶ M. Thiemens,⁷ G. Dominguez,^{7,8} A. H. Castro Neto,^{3,9} A. Zettl,^{4,5,10} F. Keilmann,¹¹ P. Jarillo-Herrero,² M. M. Fogler,¹ D. N. Basov^{1*}

van der Waals heterostructures assembled from atomically thin crystalline layers of diverse two-dimensional solids are emerging as a new paradigm in the physics of materials. We used infrared nanoimaging to study the properties of surface phonon polaritons in a representative van der Waals crystal, hexagonal boron nitride. We launched, detected, and imaged the polaritonic waves in real space and altered their wavelength by varying the number of crystal layers in our specimens. The measured dispersion of polaritonic waves was shown to be governed by the crystal thickness according to a scaling law that persists down to a few atomic layers. Our results are likely to hold true in other polar van der Waals crystals and may lead to new functionalities.

Layered van der Waals (vdW) crystals consist of individual atomic planes weakly coupled by vdW interaction, similar to

graphene monolayers in bulk graphite (1–3). These materials can harbor superconductivity (2) and ferromagnetism (4) with high transition

temperatures, emit light (5, 6), and exhibit topologically protected surface states (7), among many other effects (8). An ambitious practical goal (9) is to exploit atomic planes of vdW crystals as building blocks of more complex artificially stacked structures where each such block will deliver layer-specific attributes for the purpose of their combined functionality (3). We explored the behavior of phonon polaritons in hexagonal boron nitride (hBN), a representative vdW crystal. The phonon polaritons are collective modes that originate from coupling of photons with optical phonons (10) in polar crystals. They have been investigated in the context of energy transfer (11, 12), coherent control of the lattice (13), ultramicroscopy (14, 15), “superlensing” (16), and metamaterials (17, 18). Tunable phonon polaritons that we discovered in hBN by direct infrared (IR) nanoimaging set the stage for the implementation of these appealing concepts in vdW heterostructures. Polaritonic effects reported here are likely generic to other polar vdW solids because these materials commonly show optical phonons.

¹Department of Physics, University of California, San Diego (UCSD), La Jolla, CA 92093, USA. ²Department of Physics, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. ³Department of Physics, Boston University, Boston, MA 02215, USA. ⁴Department of Physics, University of California, Berkeley, Berkeley, CA 94720, USA. ⁵Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. ⁶National Institute for Materials Science, Namiki 1-1, Tsukuba, Ibaraki 305-0044, Japan. ⁷Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA. ⁸Department of Physics, California State University, San Marcos, San Marcos, CA 92096, USA. ⁹Graphene Research Centre and Physics Department, National University of Singapore, 6 Science Drive 2, Singapore 117546, Singapore. ¹⁰Kavli Energy NanoSciences Institute at the University of California, Berkeley, and the Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. ¹¹Ludwig-Maximilians-Universität and Center for Nanoscience, 80539 München, Germany.

*Corresponding author. E-mail: dbasov@physics.ucsd.edu

hBN stands out in this class of materials thanks to its light constituent elements, which yield particularly strong phonon resonances that span a broad region of the technologically important IR band.

IR nanoimaging and Fourier transform IR nano-spectroscopy (nano-FTIR) experiments were performed at UCSD by using a scattering-type scanning near-field optical microscope (s-SNOM) (19). The physics of polariton imaging using s-SNOM is akin to nanoimaging of surface plasmons (20, 21) (Fig. 1A). In short, we illuminated the metalized tip of an atomic force microscope (AFM) with an IR beam. We used quantum cascade lasers (QCLs) with tunable frequency $\omega = 1/\lambda_{\text{IR}}$, where λ_{IR} is IR beam wavelength and a broad-band difference frequency generation (DFG) laser system (22). Our AFM tip with curvature radius $a \approx 25$ nm is polarized by the incident IR beam. The light momenta imparted by the tip extend to the typical range of momenta supporting phonon polaritons in hBN (Fig. 2E). Therefore, the strong electric field between the tip and the sample provides the necessary momentum to launch polariton waves of wavelength λ_p that propagate radially outward from the tip along the hBN surface. AFM tips exploited in our nanospectroscopy instrument are commonly referred to as optical antennas (23): an analogy that is particularly relevant to describe the surface wave launching function of the tip. Upon reaching the sample edge, polaritonic waves are reflected back, forming a standing wave between the tip and hBN edge. As the tip is scanned toward the edge, the scattering signal collected from underneath the tip reveals oscillations with the period of $\lambda_p/2$.

Representative nanoimaging data are displayed in Fig. 1, B and D to F, where we plot the normalized near-field amplitude $s(\omega) = s_{\text{hBN}}(\omega)/s_{\text{Au}}(\omega)$ at several IR frequencies in the 1550- to 1580- cm^{-1} range. Here, $s_{\text{hBN}}(\omega)$ and $s_{\text{Au}}(\omega)$ are the scatter-

ing amplitudes for, respectively, the sample and the reference (Au-coated wafer) (19). The amplitudes were demodulated at the third or the fourth harmonic of the tapping frequency to isolate the genuine near-field signal (23). The images in Fig. 1, B to F, were taken for a tapered hBN crystal of thickness $d = 256$ nm. They reveal a hatched pattern of periodic maxima, fringes, of $s(\omega)$ running parallel to the edges, with the “hot spots” located where two or more fringes intersect. We observed similar fringe patterns in other hBN samples, including those that are only a few atomic layers thick (Fig. 1G). Such patterns are readily accounted for (Fig. 1C) within a phenomenological theory that considers reflections from the tapered edges (19).

Data in Fig. 1 allow one to obtain the polariton wavelength λ_p simply by doubling the fringe period, and the corresponding momentum can be calculated as $q = 2\pi/\lambda_p$. We used two approaches to determine the dispersion relation $q = q(\omega)$ of the polaritons. One method (15, 20, 21) is to analyze the periodicity of fringes at discrete frequencies of the IR source (Fig. 1, B and D to G). We have complemented this procedure with a technique capable of capturing the entire dispersion in the course of one single scan of our nanoscope. We executed this line scan on an hBN crystal with a large surface area to ensure that the $L = 0$ boundary is the principal reflector for the tip-launched polaritons (Fig. 2A). Such a line scan (Fig. 2, A, B, and D) is composed of a series of broad-band nano-FTIR spectra taken at every pixel. Starting in the region of unobscured SiO_2 substrate ($L < 0$) and continuing through the hBN crystal ($L > 0$), we combined the spectra from all pixels along the line scan and thus obtained a two-dimensional map $s(L, \omega)$, shown in Fig. 2B. In the plot, we observed a series of resonances that systematically vary with frequency ω and the distance from the sample edge

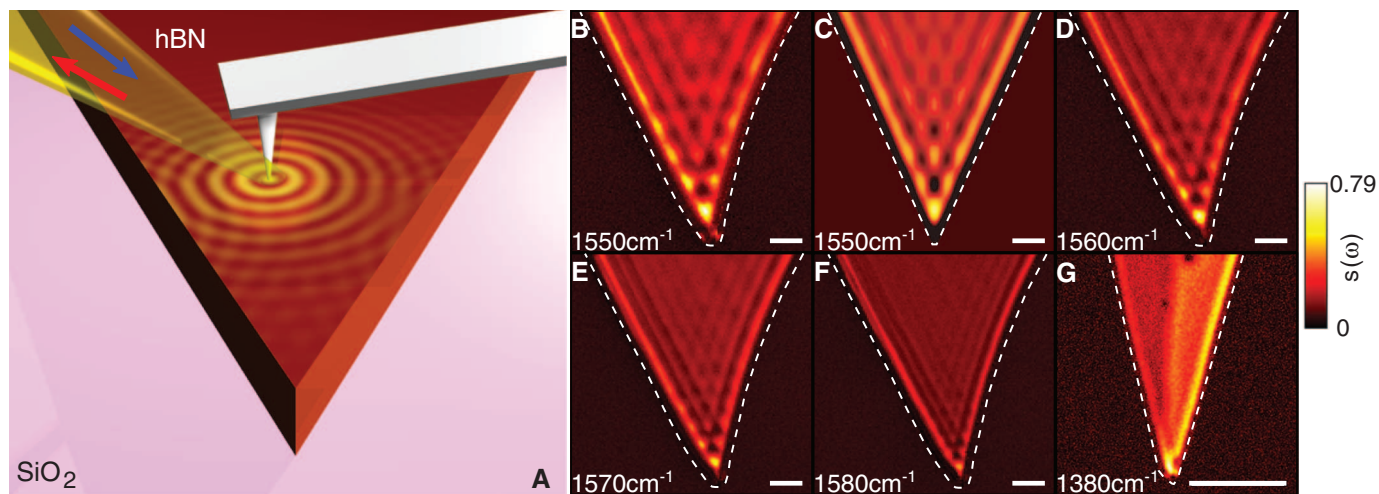


Fig. 1. Real-space imaging of surface phonon polaritons on hBN. (A) Schematics. Arrows denote the incident and back-scattered IR light. Concentric yellow circles illustrate the phonon polariton waves launched by the AFM tip and reflected by the two edges of a tapered hBN crystal. (B and D to F) IR near-field images of the normalized amplitude $s(\omega)$ defined in the text

and taken at different IR frequencies [hBN thickness in (B) to (F) $d = 256$ nm]. (C) Simulation of the phonon polariton interference pattern (19). (G) Phonon polaritons probed in three-layer (left) and four-layer (right) hBN crystals. White dashed line tracks the hBN edges according to the AFM topography. Scale bars indicate 800 nm.

L . The nano-FTIR spectra from three representative positions are shown in Fig. 2C. Each of the frames in Fig. 2C and each pixel in Fig. 2B unveil phonon polaritons in the frequency domain. The momentum q corresponding to each ω in this map can be found from the fringe periodicity along the $\omega = \text{constant}$ cut. Therefore, a single line scan is sufficient to extract the complete dispersion profile of any surface mode.

The two approaches for mapping the surface wave dispersion produced consistent results (triangles in Fig. 2B were obtained from monochromatic imaging). The broad-band line scan data (dots in Fig. 2E) allowed us to probe the dispersion in the $\omega - q$ parameter space (1430 to 1530 cm^{-1}) that cannot be investigated through the single-frequency imaging because of unavailability of proper QCLs. The experimental data for phonon polariton dis-

persion in Fig. 2E are in excellent agreement with the modeling results. Briefly, the surface polaritons correspond to the divergences of the reflectivity $r_p(q + i\kappa, \omega)$ of the system at complex momenta $q + i\kappa$ (10). For $\lambda_p \ll \lambda_{\text{IR}}$, we derived the analytical formula for polariton dispersion (19):

$$q(\omega) + i\kappa(\omega) = -\frac{\psi}{d} \left[\arctan\left(\frac{\epsilon_a}{\epsilon_{\perp}\psi}\right) + \arctan\left(\frac{\epsilon_s}{\epsilon_{\perp}\psi}\right) + \pi l \right],$$

$$\psi = \frac{\sqrt{\epsilon_{\parallel}}}{i\sqrt{\epsilon_{\perp}}} \quad (1)$$

where $\epsilon_a(\omega)$, $\epsilon_{\perp}(\omega)$, $\epsilon_{\parallel}(\omega)$, and $\epsilon_s(\omega)$ are the dielectric functions of air, hBN (for directions

perpendicular and parallel to the c axis), and SiO_2 substrate, respectively. The propagating modes correspond only to those integer l (if any) for which the loss factor $\gamma = \alpha\kappa/q$ is positive and less than unity. Parameter $\alpha = \pm$ is the sign of the group velocity $d\omega/dq$ (19). An instructive way to visualize both the dispersion and the damping is via a false-color plot of $\text{Im } r_p(q, \omega)$ (19, 24) at real q and ω (Fig. 2E). Our data line up with the topmost of these curves, which corresponds to the principal $l = 0$ branch (19) in Eq. 1.

Additional insights into the photonic and polaritonic properties of hBN were obtained by analyzing the frequency dependence of the nano-FTIR spectra. We collected the spectrum in Fig. 2F far away from the hBN edges, where the surface waves are damped and the scattering amplitude signal is solely governed by the

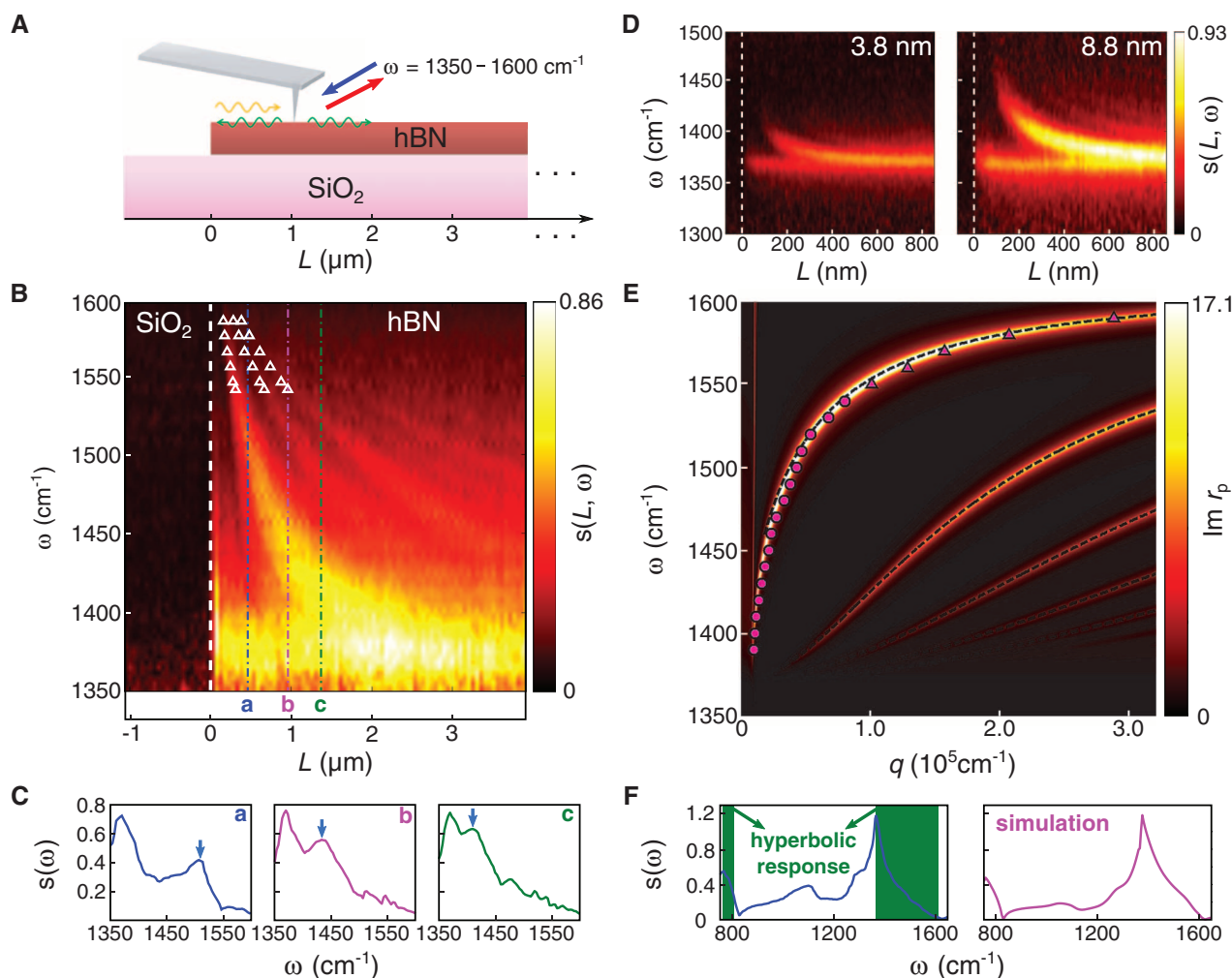


Fig. 2. The surface phonon polariton dispersion and nano-FTIR spectra. (A) Schematics of a nano-FTIR line scan across the hBN crystal. Arrows denote the incident and back-scattered IR beam spanning 1350 to 1600 cm^{-1} . Polaritonic waves are launched (green) by AFM tip and then reflected (orange) by hBN edge at $L = 0$. **(B)** Polaritonic features detected in a single line scan in (A). The normalized scattering amplitude spectra $s(\omega)$ is plotted in the false color scale. White dashed line at $L = 0$ marks the edge of the hBN crystal (thickness $d = 134$ nm). Triangles, fringe maxima extracted from monochromatic imaging similar to Fig. 1. **(C)** Nano-FTIR spectra at three representative locations along the line scan marked in (B). The

peaks marked by the arrows correspond to the dominant polariton interference fringe. **(D)** Phonon polariton features as probed via line scans for ultrathin hBN crystals with $d = 3.8$ nm (left) and $d = 8.8$ nm (right). **(E)** The dispersion relation of phonon polaritons in hBN. Triangles indicate data from monochromatic imaging in Fig. 1; dots, the nano-FTIR results from (B). The data are superimposed on a false-color plot of calculated $\text{Im } r_p$ (19); the black dashed lines are from Eq. 1. The straight line on the left represents the light line. **(F)** Nano-FTIR spectrum $s(\omega)$ for the hBN crystal (Fig. 1, A to F) taken away from the sample edges. The solid (green) part of the data corresponds to hBN's hyperbolic region where $\text{Re}\epsilon_{\perp} \cdot \text{Re}\epsilon_{\parallel} < 0$.

local interaction with the phonon resonances (25). Two of these resonances centered around 770 and 1370 cm^{-1} are due to the c axis and the in-plane phonon modes of hBN, respectively (26, 27). The hump-dip feature around 1100 cm^{-1} originates from the SiO_2 substrate (28): a consequence of a partial transparency of our specimen. The quantitative relation between this spectrum, the reflectivity $r_p(q, \omega)$, and the fundamental phonon modes can be established by numerical modeling of the tip-sample interaction (19). The right plot of Fig. 2F indicates that our model captures the gross features of the data. Moreover, the hBN is an example of a natural hyperbolic material (29): a crystal possessing the in-plane and out-of-plane components of the dielectric tensor having the opposite signs so that $\text{Re}\epsilon_{\perp} \cdot \text{Re}\epsilon_{\parallel} < 0$. Hyperbolic regions are marked in green in Fig. 2F.

The layered nature of vdW materials, including hBN, facilitates the control of both the wavelength and the amplitude of polaritonic waves by varying the thickness d of the specimens. Representative line profiles (Fig. 3A) for specimens with d in the range of 150 to 250 nm were taken normal to the crystal edge at $L = 0$. The thickness was measured simultaneously with the scattering amplitude through the AFM topography.

All fringe profiles share the same line form with a prominent peak close to the edge followed by weaker peaks that are gradually suppressed away from the edge. The oscillation period, equal to $\lambda_p/2$ (arrows in Fig. 3A), systematically decreases as the samples become thinner. This scaling extends down to a few atomic layers (Fig. 3, B and C).

The measured polariton wavelength (Fig. 3E) agrees with the theoretical predictions (Fig. 3D). For λ_p smaller than about one-half of $\lambda_{\text{IR}} = 7.1 \mu\text{m}$, the polariton wavelength scales linearly with the crystal thickness d , in agreement with Eq. 1; at larger λ_p , the linear law shows signs of saturation, also in accord with our model (Fig. 3E inset). Experimentally, the phonon polaritons display thickness-tunability persisting down to three atomic layers (Fig. 3, B and C). We detected polaritons in even thinner samples (bilayer and monolayer hBN). However, the quantitative analysis of these latter data is complicated because of the increasing role of the substrate in the polaritonic response that calls for further experiments on suspended membranes.

Similar to surface plasmons, the phonon polaritons allow one to confine and control electromagnetic energy at the nanoscale (30). In

fact, the line form in Fig. 3A strongly resembles plasmonic standing waves in graphene (20, 21). The confinement factor $\lambda_{\text{IR}}/\lambda_p$ reaches 25 in hBN, comparable to that of plasmons in graphene (20, 21). Yet these compact polaritons in hBN are able to travel at least 5 to 10 μm , compared with less than 0.5 μm for graphene plasmons. The corresponding loss factor $\gamma = \alpha\kappa/q$ is around 0.055, much smaller than a typical γ in graphene. The low damping of polaritons in our insulating samples is consistent with the absence of the electronic losses, the dominant damping channel in plasmonics. The observed losses can likely be further suppressed by improving the crystallographic order of the crystals.

Data in Figs. 1 to 3 show that phonon polaritons of the desired wavelength and confinement can be engineered by varying the number of atomic layers in hBN by, for example, exfoliation techniques. Thus, hBN and likely other polar layered materials can be integrated into vdW heterostructures (3) to serve not only as electrically insulating spacers but also as waveguides for weakly damped polaritons capable of traveling over considerable distances. Additionally, the hyperbolic response of few-layer hBN is appealing in the context of unique nanophotonics characteristics of this class of solids (29).

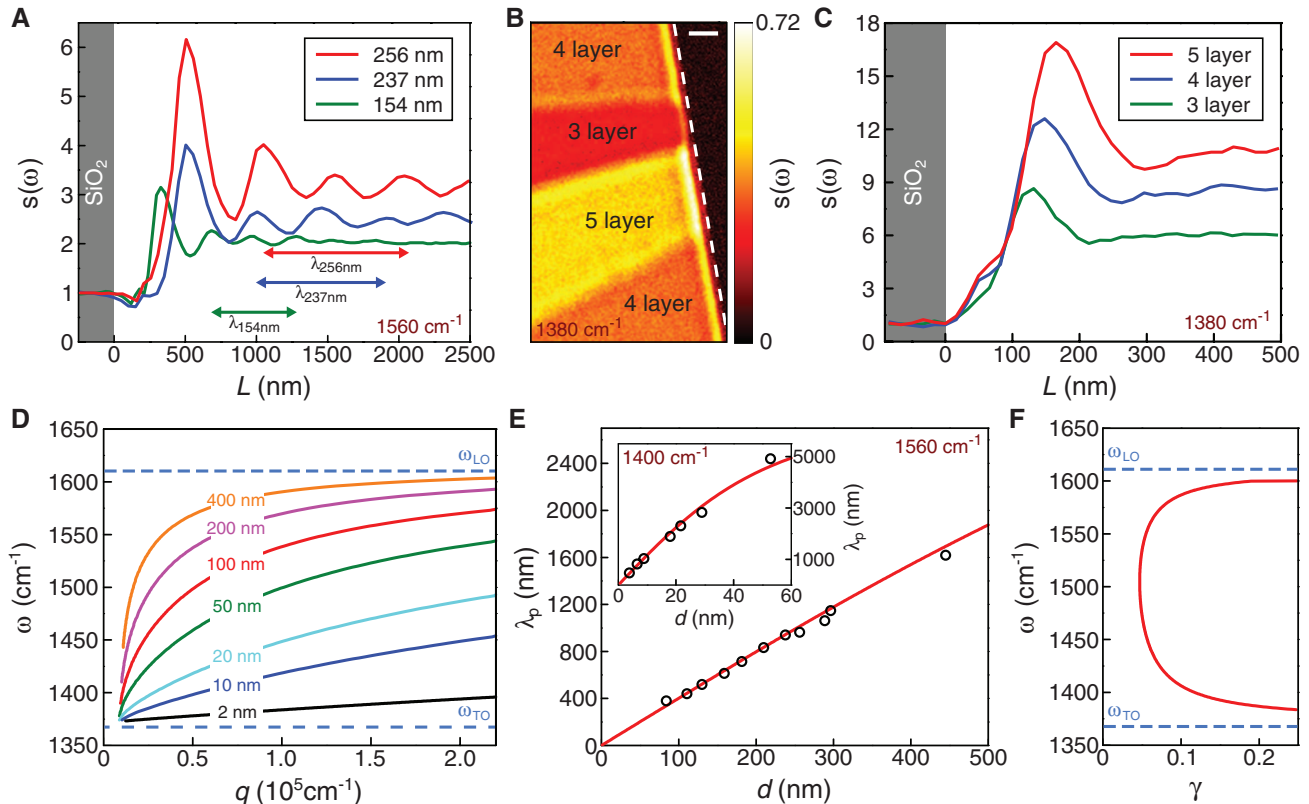


Fig. 3. The evolution of the phonon polariton wavelength and amplitude with the thickness of hBN crystals. (A) Line profiles of the scattering amplitude $s(\omega)$ at 1560 cm^{-1} for hBN crystals with $d = 154, 237$, and 256 nm . Arrows indicate the polariton wavelength. (B) Near-field image and (C) phonon polariton line profiles for few-layer hBN crystals. White dashed line in (B) tracks the sample boundary; scale bar in (B), 400 nm . (D) Cal-

culated dispersion relation of the $l = 0$ branch of the phonon polaritons in hBN for various crystal thicknesses (d). TO and LO frequencies are marked with blue dashed lines. (E) Dots, wavelengths of phonon polaritons probed at 1560 cm^{-1} for crystals with different thicknesses (d); red line, calculated thickness-dependence relation. (Inset) Thickness-dependence relation probed at 1400 cm^{-1} for ultrathin hBN crystals. See (19) for details. (F) Calculated loss factor for phonon polaritons.

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Rapid Reductions in North Atlantic Deep Water During the Peak of the Last Interglacial Period

Eirik Vinje Galaasen,^{1*} Ulysses S. Ninnemann,^{1,2} Nil Irvali,² Helga (Kikki) F. Kleiven,^{1,2} Yair Rosenthal,³ Catherine Kissel,⁴ David A. Hodell⁵

Deep ocean circulation has been considered relatively stable during interglacial periods, yet little is known about its behavior on submillennial time scales. Using a subcentennially resolved epibenthic foraminiferal $\delta^{13}\text{C}$ record, we show that the influence of North Atlantic Deep Water (NADW) was strong at the onset of the last interglacial period and was then interrupted by several prominent centennial-scale reductions. These NADW transients occurred during periods of increased ice rafting and southward expansions of polar water influence, suggesting that a buoyancy threshold for convective instability was triggered by freshwater and circum-Arctic cryosphere changes. The deep Atlantic chemical changes were similar in magnitude to those associated with glaciations, implying that the canonical view of a relatively stable interglacial circulation may not hold for conditions warmer and fresher than at present.

Future climate could be affected on a global scale if the circulation of North Atlantic Deep Water (NADW), the main water mass ventilating the deep Atlantic (Fig. 1) (1), is altered. Such changes could have widespread and long-lasting impacts—including, for example, on regional sea level (2), the intensity and pacing of Sahel droughts (3), and the pattern and rate of ocean acidification and CO_2 sequestration

(4). However, the response of NADW to high-latitude warming and ocean freshening, both of which would decrease source region density and potentially inhibit NADW formation, remains a key uncertainty in future climate projections. Model estimates range from nearly no change to ~50% reduction in Atlantic Meridional Overturning Circulation by 2100 CE (5). Compounding the uncertainty, models may inherently underestimate the possibility for abrupt and large changes (6), and there may even be critical stability thresholds in surface ocean buoyancy that, if crossed, could switch circulation into an equilibrium state without strong NADW formation (7, 8). The current consensus is that we are far from any such stability thresholds and that the modern style of vigorous NADW ventilation is a robust feature of warm interglacial climates. Only modest millennial-scale NADW variability has been found to occur during interglacials (9, 10) relative to

that seen during colder glacial periods (11). However, large but shorter-lived transient anomalies might be possible even in the midst of a generally vigorous interglacial circulation (7, 12, 13). Hence, reconstructions with appropriate resolution to characterize the short-term instability of NADW during warmer climates are needed to assess model fidelity and constrain possible tipping points for ocean circulation. We used deep sea sediment proxy records from key locations (Fig. 1) to assess the occurrence and magnitude of centennial-scale variability in NADW over the warm interval of the last interglacial period (LIG) [marine isotope stage (MIS) 5e]. The LIG is a useful period for evaluating the sensitivity of NADW to key features that we may face in the future, including a warmer and fresher North Atlantic than at present (14, 15) and the retreat of the circum-North Atlantic cryosphere (15, 16).

We characterized the short-term variability of NADW over the LIG using sediment core MD03-2664 (57°26.34'N, 48°36.35'W; 3442 m water depth) from the Eirik Drift site used to identify the centennial-scale NADW reduction associated with the climate anomaly 8.2 thousand years before the present (ky B.P.) (12). This site monitors the newly formed, integrated Nordic Seas overflows (12) that are the primary constituents of lower NADW (1). The high sedimentation rate (~35 cm ky^{-1}) at this location allows a multidecadal depiction of lower NADW properties and ventilation across the LIG (~30 years per 1-cm sample), which is approximately an order of magnitude greater than the previous reconstructions used to infer millennial-scale NADW stability during the LIG (10, 17).

On our age model (18), the MIS 5e “plateau” [the interval of relatively constant minimum ice volume (benthic $\delta^{18}\text{O}$)] corresponds to 116.1 to 128.0 ky. We focused our study on this interval, referring to it as the LIG. We documented NADW variability using the carbon isotopic composition

¹Department of Earth Science, University of Bergen and Bjerknes Centre for Climate Research, Allégaten 41, 5007 Bergen, Norway.

²Uni Climate, Uni Research and Bjerknes Centre for Climate Research, Bergen, Norway. ³Institute of Marine and Coastal Sciences and Department of Earth and Planetary Sciences, Rutgers University, New Brunswick, NJ, USA. ⁴Laboratoire des Sciences du Climat et de l'Environnement/Institut Pierre Simon Laplace, CEA/CNRS/UVSQ, Gif-sur-Yvette, France. ⁵Godwin Laboratory for Paleoclimate Research, Department of Earth Sciences, University of Cambridge, Cambridge, UK.

*Corresponding author. E-mail: eirik.galaasen@geo.uib.no



Rapid Reductions in North Atlantic Deep Water During the Peak of the Last Interglacial Period

Eirik Vinje Galaasen *et al.*

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Rapid Reductions in North Atlantic Deep Water During the Peak of the Last Interglacial Period

Eirik Vinje Galaasen,^{1*} Ulysses S. Ninnemann,^{1,2} Nil Irvali,² Helga (Kikki) F. Kleiven,^{1,2} Yair Rosenthal,³ Catherine Kissel,⁴ David A. Hodell⁵

Deep ocean circulation has been considered relatively stable during interglacial periods, yet little is known about its behavior on submillennial time scales. Using a subcentennially resolved epibenthic foraminiferal $\delta^{13}\text{C}$ record, we show that the influence of North Atlantic Deep Water (NADW) was strong at the onset of the last interglacial period and was then interrupted by several prominent centennial-scale reductions. These NADW transients occurred during periods of increased ice rafting and southward expansions of polar water influence, suggesting that a buoyancy threshold for convective instability was triggered by freshwater and circum-Arctic cryosphere changes. The deep Atlantic chemical changes were similar in magnitude to those associated with glaciations, implying that the canonical view of a relatively stable interglacial circulation may not hold for conditions warmer and fresher than at present.

Future climate could be affected on a global scale if the circulation of North Atlantic Deep Water (NADW), the main water mass ventilating the deep Atlantic (Fig. 1) (1), is altered. Such changes could have widespread and long-lasting impacts—including, for example, on regional sea level (2), the intensity and pacing of Sahel droughts (3), and the pattern and rate of ocean acidification and CO_2 sequestration

(4). However, the response of NADW to high-latitude warming and ocean freshening, both of which would decrease source region density and potentially inhibit NADW formation, remains a key uncertainty in future climate projections. Model estimates range from nearly no change to ~50% reduction in Atlantic Meridional Overturning Circulation by 2100 CE (5). Compounding the uncertainty, models may inherently underestimate the possibility for abrupt and large changes (6), and there may even be critical stability thresholds in surface ocean buoyancy that, if crossed, could switch circulation into an equilibrium state without strong NADW formation (7, 8). The current consensus is that we are far from any such stability thresholds and that the modern style of vigorous NADW ventilation is a robust feature of warm interglacial climates. Only modest millennial-scale NADW variability has been found to occur during interglacials (9, 10) relative to

that seen during colder glacial periods (11). However, large but shorter-lived transient anomalies might be possible even in the midst of a generally vigorous interglacial circulation (7, 12, 13). Hence, reconstructions with appropriate resolution to characterize the short-term instability of NADW during warmer climates are needed to assess model fidelity and constrain possible tipping points for ocean circulation. We used deep sea sediment proxy records from key locations (Fig. 1) to assess the occurrence and magnitude of centennial-scale variability in NADW over the warm interval of the last interglacial period (LIG) [marine isotope stage (MIS) 5e]. The LIG is a useful period for evaluating the sensitivity of NADW to key features that we may face in the future, including a warmer and fresher North Atlantic than at present (14, 15) and the retreat of the circum-North Atlantic cryosphere (15, 16).

We characterized the short-term variability of NADW over the LIG using sediment core MD03-2664 (57°26.34'N, 48°36.35'W; 3442 m water depth) from the Eirik Drift site used to identify the centennial-scale NADW reduction associated with the climate anomaly 8.2 thousand years before the present (ky B.P.) (12). This site monitors the newly formed, integrated Nordic Seas overflows (12) that are the primary constituents of lower NADW (1). The high sedimentation rate (~35 cm ky^{-1}) at this location allows a multidecadal depiction of lower NADW properties and ventilation across the LIG (~30 years per 1-cm sample), which is approximately an order of magnitude greater than the previous reconstructions used to infer millennial-scale NADW stability during the LIG (10, 17).

On our age model (18), the MIS 5e “plateau” [the interval of relatively constant minimum ice volume (benthic $\delta^{18}\text{O}$)] corresponds to 116.1 to 128.0 ky. We focused our study on this interval, referring to it as the LIG. We documented NADW variability using the carbon isotopic composition

¹Department of Earth Science, University of Bergen and Bjerknes Centre for Climate Research, Allégaten 41, 5007 Bergen, Norway.

²Uni Climate, Uni Research and Bjerknes Centre for Climate Research, Bergen, Norway. ³Institute of Marine and Coastal Sciences and Department of Earth and Planetary Sciences, Rutgers University, New Brunswick, NJ, USA. ⁴Laboratoire des Sciences du Climat et de l'Environnement/Institut Pierre Simon Laplace, CEA/CNRS/UVSQ, Gif-sur-Yvette, France. ⁵Godwin Laboratory for Paleoclimate Research, Department of Earth Sciences, University of Cambridge, Cambridge, UK.

*Corresponding author. E-mail: eirik.galaasen@geo.uib.no

($\delta^{13}\text{C}$) of bottom water, a widely used tracer to delimit the relative influence of low-nutrient (high- $\delta^{13}\text{C}$) NADW and nutrient-rich (low- $\delta^{13}\text{C}$) Southern Ocean-sourced bottom waters (19) (Fig. 1). We reconstructed bottom water $\delta^{13}\text{C}$ using the epibenthic foraminifera *Cibicides wuellerstorfi*.

Peak epibenthic foraminifera $\delta^{13}\text{C}$ values from Eirik Drift core MD03-2664 gradually increased during the LIG by ~ 0.5 per mil (‰) (Fig. 2), paralleling long-term trends in planktonic and benthic $\delta^{13}\text{C}$ records from the Nordic Seas (20, 21) source region for NADW. The similar trends in deep water $\delta^{13}\text{C}$ at the Eirik Drift and Nordic Seas indicate a strong influence of Nordic Seas-sourced deep water at the core site during much of the LIG. This suggests that a water mass distribution similar to that of the modern Atlantic, with strong NADW influence, was established after the penultimate deglaciation and persisted on millennial and longer time scales throughout the LIG, as previously observed (10, 17).

Despite this apparent millennial-scale stability, large but short-lived $\delta^{13}\text{C}$ decreases indicate that prominent changes in bottom-water chemistry occurred on shorter time scales (Fig. 2). During these multicentennial shifts, epibenthic $\delta^{13}\text{C}$ decreased abruptly by up to ~ 0.7 ‰, an amplitude similar to that of glacial-interglacial (19) and millennial-scale (Dansgaard-Oeschger) changes in the deep Atlantic (11). By comparison, the only $\delta^{13}\text{C}$ decrease of similar magnitude found at this site during the Holocene occurred after the freshwater outburst from proglacial Lake Agassiz (12). Thus, we documented multiple LIG deep water events similar in $\delta^{13}\text{C}$ magnitude and duration to that associated with the 8.2 ky B.P. event.

What drove these transient changes in deep Atlantic chemistry? There are indications that similar bottom-water $\delta^{13}\text{C}$ reductions occur at deep sites in the subpolar North Atlantic (22) and sub-Antarctic Atlantic (23) (Fig. 3). This wide geographic distribution (Fig. 1) requires a mechanism able to reduce deep $\delta^{13}\text{C}$ at widespread locations and eliminate north-south $\delta^{13}\text{C}$ gradients in the abyssal Atlantic. We rule out changes in preformed NADW $\delta^{13}\text{C}$ as an explanation because (i) there is no evidence for such $\delta^{13}\text{C}$ changes in Nordic Seas source waters during the LIG (20, 21), (ii) the changes are smaller in sites most directly influenced by the Nordic Seas overflows (22), and (iii) it would require source water $\delta^{13}\text{C}$ changes that were larger and faster than those caused by the recent input of anthropogenic CO_2 (24). Instead, the pattern of bottom-water chemical and circulation changes suggests that the Nordic Seas overflows repeatedly decreased in density and/or shoaled to depths shallower than the Eirik Drift during the low- $\delta^{13}\text{C}$ excursions. The reduced bottom current vigor observed during the largest $\delta^{13}\text{C}$ minimum at ~ 124 ky B.P. (Fig. 3) is consistent with reduced NADW production (25). Sudden incursions of Southern Source Water (SSW) as NADW waned would explain the abrupt (multidecadal) onset of the anomalies as well as the convergence of bottom-water $\delta^{13}\text{C}$ toward

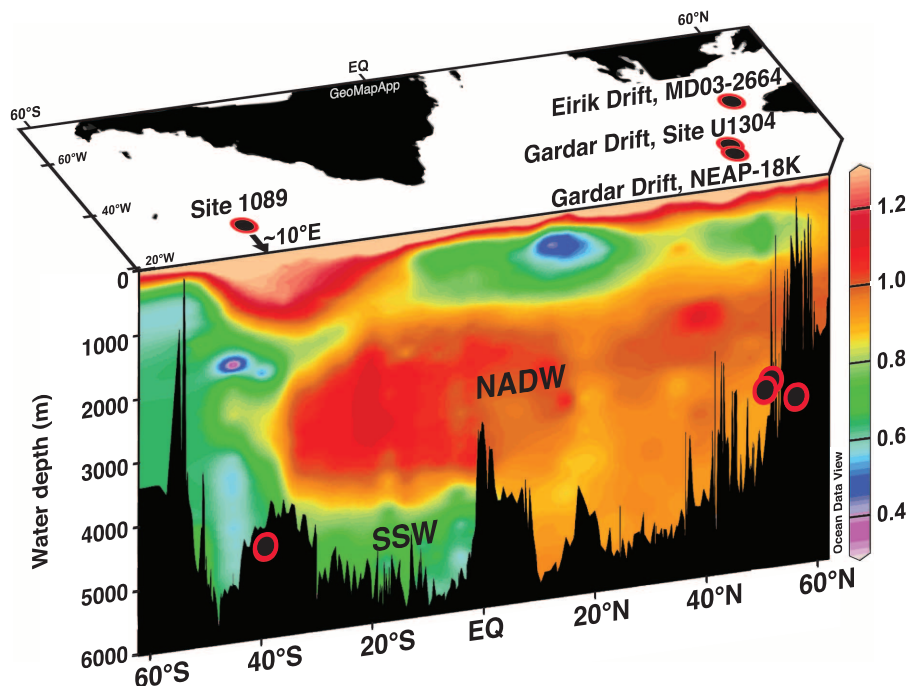
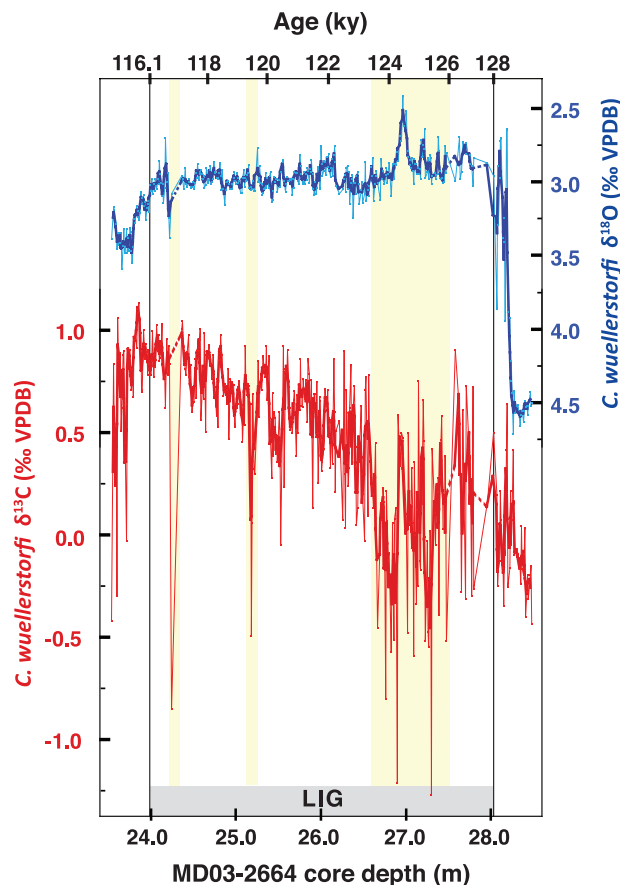


Fig. 1. Locations of core sites. MD03-2664 (57°26'N, 48°36'W; 3442 m), IODP Site U1304 (53°03'N, 33°32'W; 3082 m), NEAP-18K (52°46'N, 30°21'W; 3275 m), and ODP Site 1089 (40°56'S, 9°54'E; 4624 m) (core specifics are summarized in table S1). Core locations are projected onto a north-south section of dissolved inorganic carbon $\delta^{13}\text{C}$ (‰) in the Atlantic (World Ocean Circulation Experiment A16) (36). High- $\delta^{13}\text{C}$ (low-nutrient) NADW occupies the basin at ~ 2 to 4 km water depth, overlying (high-nutrient) low- $\delta^{13}\text{C}$ SSW, corresponding to modern Antarctic Bottom Water.

Fig. 2. Deep-water property changes during the LIG. Plot of epibenthic *C. wuellerstorfi* $\delta^{18}\text{O}$ (blue) and $\delta^{13}\text{C}$ records (red) with three-point running means (bold lines) from the Eirik Drift (core MD03-2664) versus core depth (bottom) and age (top). VPDB, Vienna Pee Dee belemnite standard. The horizontal gray bar and vertical lines denote the LIG (defined here as the MIS 5e isotopic plateau), and yellow shading denotes LIG periods of pronounced bottom-water $\delta^{13}\text{C}$ reductions. Dashed lines indicate intervals with insufficient foraminifera for analysis. *C. wuellerstorfi* $\delta^{13}\text{C}$ values of ~ 0.5 ‰ (early LIG) to ~ 1.0 ‰ (late LIG) are similar to preformed Nordic Seas values (20), indicating NADW influence.



SSW values ($\leq 0.0\%$) (26) (Fig. 3). Likewise, shoaling and a reduced influx of NADW to the Southern Ocean could explain the decrease in deep circumpolar $\delta^{13}\text{C}$ (Fig. 3).

Changes in the production or density of NADW are likely to be linked to changes in North Atlantic source conditions (7, 8). During the LIG, the high northern latitudes were warmer than they are today, and the circum-Arctic marine and terrestrial cryospheres were smaller (15, 16). Models suggest that increased North Atlantic surface buoyancy, due to relative warmth and increased freshwater fluxes from the LIG ice mass retreat, could have inhibited convection and delayed the onset of NADW formation (14, 15). Our results provide an alternative view of deep ocean behavior. Rather than a general suppression of NADW production, our records suggest that relatively brief, intermittent NADW reductions occurred against a background of strong NADW ventilation that was established at the onset of the LIG. This implies a fundamentally different deep ocean behavior, similar to what one might expect from threshold-like flickering of deep convection.

The largest and longest NADW anomalies occur early in the LIG when ice melting was

strongest (15, 16, 27) and residual ice masses from the prior glaciation persisted (28). This pattern is reminiscent of the deep ocean and cryosphere coupling apparent in the early Holocene (12, 13). Supporting a connection between ice mass retreat and the NADW reductions, Eirik Drift ice-rafted debris (IRD) deposition and high *Neogloboquadrina pachyderma* (sinistral) Ba/Ca ratios indicate that both iceberg supply and meltwater input persisted during the early LIG (Fig. 4). High Ba/Ca ratios in *N. pachyderma* (s) tests indicate the presence of Ba-rich glacial meltwater inputs (29). The increased number of NADW anomalies during the early LIG relative to the Holocene may reflect generally fresher North Atlantic conditions (14, 15), due in part to the greater Greenland Ice Sheet retreat (16, 27). IRD input and Ba/Ca ratios tapered off at the Eirik Drift after ~ 123.5 and ~ 124 ky, respectively, suggesting that the stabilization of NADW was coincident with the reduced input of icebergs and glacial meltwater to the North Atlantic (Fig. 4).

Further supporting a role for freshwater forcing in triggering the deep-water anomalies, an outburst flood event analogous to that associated with the 8.2 ky B.P. event occurred during the early LIG (28). The detrital layer found throughout the

Labrador Sea that is associated with this outburst flood and a marked surface freshening event early in the LIG (18, 28) is also present in our core at ~ 124.5 ky (18) and is followed immediately by the largest and longest NADW reduction of the LIG. This coincidence supports a role for surface buoyancy changes in altering deep Atlantic water mass geometries and bottom-water chemistry. However, it is important to note that the existence of NADW reductions during the second half of the LIG (at ~ 116.8 and ~ 119.5 ky; Fig. 3), when conditions favored ice mass expansion rather than rapid retreat (30), suggests that decaying ice sheets may not be the sole means of triggering transient NADW reductions. Warming and freshening related to hydrologic changes, as in model simulations of future warming (5), could also have increased buoyancy and reduced NADW during the LIG.

The large anomalies in NADW distribution allow us to assess the relationship between climate and deep ocean circulation changes during interglacial periods. Ocean circulation-driven changes in interhemispheric heat transport could explain the inverse long-term Greenland and Antarctic temperature trends over the early LIG (30). Likewise, climate was more variable during the LIG than the Holocene (20, 21), suggesting a possible link with the increased NADW variability seen in our records. However, evidence for the type of widespread cooling associated with the early Holocene (8.2 ky B.P.) event (31) is lacking for the LIG events. This may indicate that sea ice feedbacks, which would be reduced during warmer interglacials, are critical for amplifying the climate response to NADW changes (32). Alternatively, it may simply reflect the difficulty of resolving and dating such short-lived LIG events in the proxy records that are currently available. Regardless of the scale of the climate response, Eirik Drift surface proxy reconstructions suggest that a distinct pattern of surface-ocean changes accompanied the NADW reductions. Increases in the abundance of the polar planktonic foraminifera *N. pachyderma* (s) document greater polar water influence in the northwest Atlantic during each NADW reduction (Fig. 4). These polar waters were fresher [marked by lower *N. pachyderma* (s) $\delta^{18}\text{O}$] during the early LIG than in the late LIG (Fig. 4), probably reflecting the early high-latitude freshening (33). This pattern of southward-displaced polar waters associated with NADW reductions mimics the millennial-scale pattern observed throughout the last glacial cycle (34), suggesting that similar ocean-atmosphere dynamics operated during interglacial centennial-scale events. A comparable pattern of changes, though briefer and subtler in character, also marked the Great Salinity Anomaly in the 1960s, when the expansion of fresh polar waters caused cessation of Labrador Sea convection (35).

Our results call for a reevaluation of the notion that the deep Atlantic ventilation is relatively stable and vigorous during interglacial periods.

Fig. 3. North and South Atlantic deep-water proxy records spanning the LIG (116.1 to 128.0 ky):

(A) IODP Site U1304 with a three-point running mean after (22), **(C)** MD03-2664 with a three-point running mean, and **(D)** ODP Site 1089 after (23); and **(B)** NEAP-18K sortable silt mean grain sizes after (25). Yellow shading marks the interval of high-amplitude variability in all records. Dashed lines in (C) indicate intervals with insufficient foraminifera for analysis.

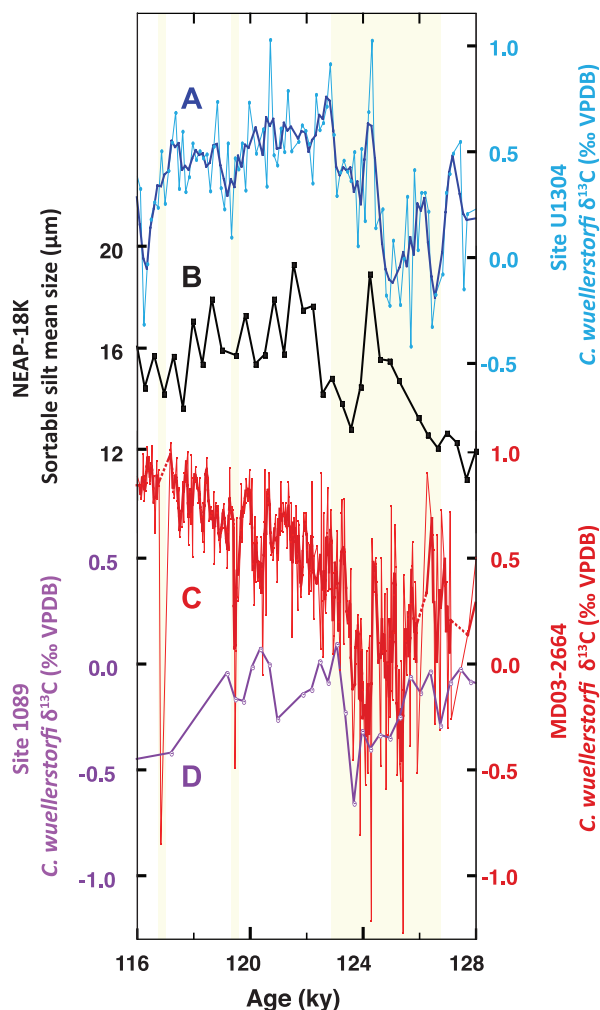
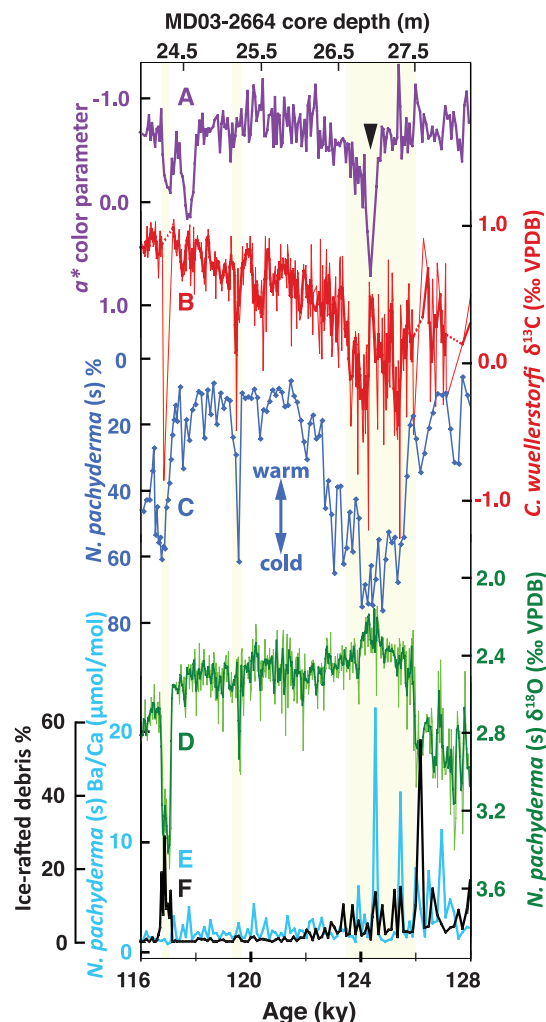


Fig. 4. Proxy records spanning the LIG (116.1 to 128.0 ky) section of core MD03-2664. (A) Red-green color parameter a^* , **(B)** *C. wuellerstorfi* $\delta^{13}\text{C}$ with a three-point running mean, **(C)** *N. pachyderma* (s) abundance (% of assemblage), **(D)** *N. pachyderma* (s) $\delta^{18}\text{O}$ with a three-point running mean, **(E)** *N. pachyderma* (s) Ba/Ca, ($\mu\text{mol/mol}$), and **(F)** IRD (%) [(C), (D), and (F) are after Irali *et al.* (33)]. Yellow shading denotes periods of pronounced bottom-water $\delta^{13}\text{C}$ reductions. Dashed lines in (B) indicate intervals with insufficient foraminifera *C. wuellerstorfi* for analysis. The triangle in (A) denotes the position of the red detrital layer deposited during an outburst flood event (18). The (C) *N. pachyderma* (s) abundance record, a proxy for surface temperature changes in the subpolar North Atlantic region (14), indicates changes in Eirik Drift sea surface temperatures.



Our records resolving centennial-scale variability provide clear evidence for large changes in deep Atlantic water mass geometry—similar in magnitude to glacial millennial-scale events—punctuating the LIG. The most prominent NADW reductions occurred during periods of ice sheet melting and known freshwater outbursts, attesting to the importance of surface buoyancy forcing in triggering such events. Concerns about the future evolution of the thermohaline circulation have revolved around the potential existence of inherent tipping points that, if crossed, could lead to long-term perturbations in the mode of ventilation (7, 8), although the existence of such bi-stability is debated (6). The specific characteristics of the observed interglacial NADW anomalies provide insights into the deep ocean's stability and potential impact on a warming world. The shorter duration of the interglacial events as compared to their glacial equivalents indicates that either the forcing or the ocean's inherent response was more transient during warm (interglacial) conditions, arguing against an interglacial deep Atlantic with irreversible tipping points. Nevertheless, the large and rapid variability in deep ocean ventilation also supports the existence of a critical

stability threshold; most likely related to deep convection, given the apparent link to surface buoyancy forcing. Although transient in nature, large interglacial anomalies in deep ventilation may still be of particular concern. If triggered, they could alter regional climate (3) and CO_2 sequestration pathways (4) and in the most extreme cases would as much as double the sea-level increases projected by 2100 CE for densely populated circum-North Atlantic regions (2). Our results suggest that past interglacial NADW reductions were abruptly initiated, persisted for centuries, and were concentrated around periods of North Atlantic warmth and freshwater addition, both of which are expected in the future (5).

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Supplementary Materials

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Data Table S1

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Structural Basis for Heavy Metal Detoxification by an Atm1-Type ABC Exporter

Jonas Y. Lee *et al.*
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Structural Basis for Heavy Metal Detoxification by an Atm1-Type ABC Exporter

Jonas Y. Lee,¹ Janet G. Yang,¹ Daniel Zhitnitsky,² Oded Lewinson,² Douglas C. Rees^{1*}

Although substantial progress has been achieved in the structural analysis of exporters from the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, much less is known about how they selectively recognize substrates and how substrate binding is coupled to ATP hydrolysis. We have addressed these questions through crystallographic analysis of the Atm1/ABCB7/HMT1/ABCB6 ortholog from *Novosphingobium aromaticivorans* DSM 12444, NaAtm1, at 2.4 angstrom resolution. Consistent with a physiological role in cellular detoxification processes, functional studies showed that glutathione derivatives can serve as substrates for NaAtm1 and that its overexpression in *Escherichia coli* confers protection against silver and mercury toxicity. The glutathione binding site highlights the articulated design of ABC exporters, with ligands and nucleotides spanning structurally conserved elements to create adaptable interfaces accommodating conformational rearrangements during the transport cycle.

The Atm1/ABCB7/HMT1/ABCB6 family of adenosine triphosphate (ATP)-binding cassette (ABC) exporters has been implicated in transition metal homeostasis and detoxification processes (1–4). In eukaryotes, the Atm1/ABCB7 ortholog is present in the inner membrane of mitochondria and is required for the formation of cytosolic iron-sulfur cluster-containing proteins (5); mutations of this protein

result in mitochondrial accumulation of iron (6) and are responsible for X-linked sideroblastic anemia (7). ABCB6 was first identified as a porphyrin transporter present in the outer membrane of mitochondria (8), but it may also play a role in arsenic resistance (9) and is present in the Golgi (10), lysosomal (11), and plasma membranes (12). Other homologs have been implicated in heavy metal detoxification, including

cadmium resistance by heavy metal tolerance factor-1 (HMT1) in *Caenorhabditis elegans* and *Drosophila melanogaster* (13–15). The available evidence (16) suggests that for some of these transporters, the substrates may be related to the thiol-containing glutathione (GSH), a tripeptide of sequence γ -Glu-Cys-Gly. GSH is an abundant cellular antioxidant that mediates resistance to electrophiles, heavy metals, and xenobiotics (17–19).

As a starting point for addressing the molecular mechanism of this family of ABC transporters and their physiological role(s) in metal homeostasis, we have solved the structure of the Atm1-family ABC exporter from *Novosphingobium aromaticivorans* (NaAtm1) at 2.4 Å resolution (20). NaAtm1 shares ~45% sequence identity with *Saccharomyces cerevisiae* Atm1, human ABCB7, and HMT-1 (fig. S1), and hence it should provide a useful model for these eukaryotic transporters [see Srinivasan *et al.* (21)]. Reflecting the basic architecture of the ABC exporter family that was first elucidated for the Sav1866 transporter (22) and subsequently found in other ABC exporters (23–27), NaAtm1 forms a homodimer, with each subunit containing six transmembrane

¹Howard Hughes Medical Institute and Division of Chemistry and Chemical Engineering, Mail Code 114-96, California Institute of Technology, Pasadena, CA 91125, USA. ²Department of Microbiology, Rappaport Family Institute for Research, Faculty of Medicine, Technion-Israel Institute of Technology, 32000 Haifa, Israel.

*Corresponding author. E-mail: dcrees@caltech.edu

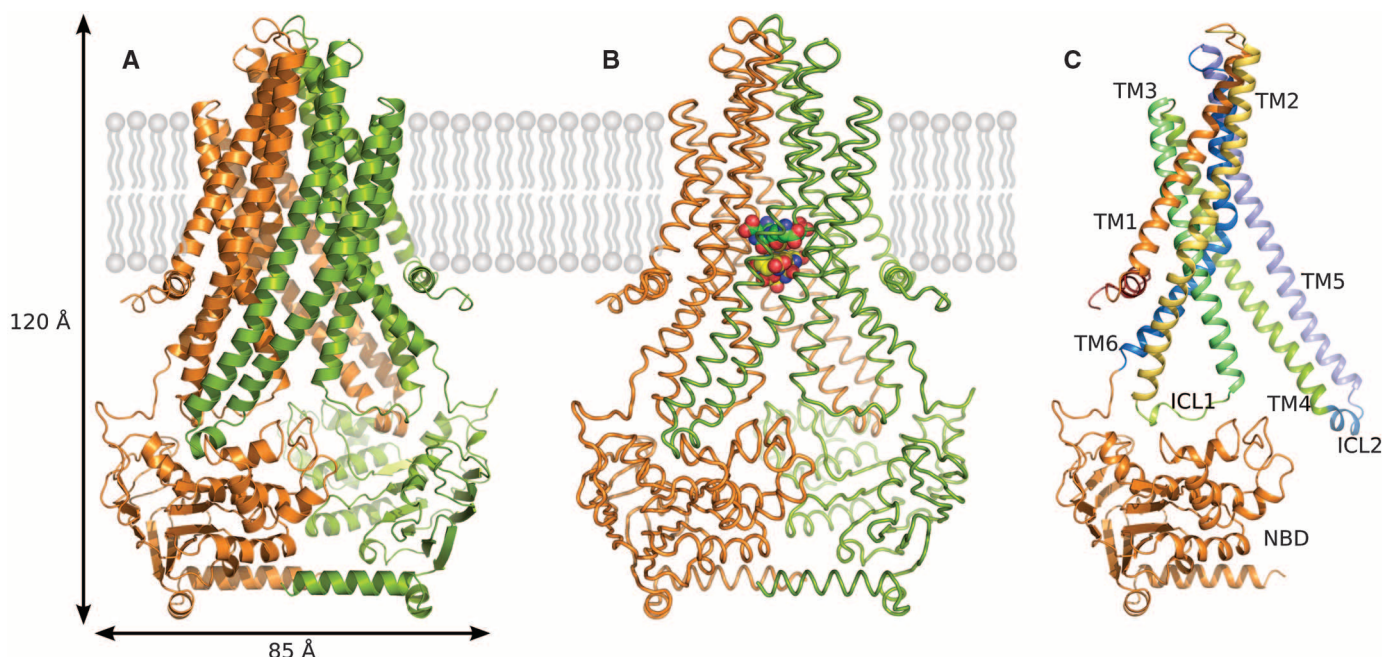


Fig. 1. Structural representations of NaAtm1. (A) Ribbon diagram illustrating the dimeric structure of NaAtm1, viewed normal to the molecular two-fold axis with the membrane-spanning domains and NBDs oriented toward the top and bottom, respectively. The approximate position of the membrane is designated by the gray bilayer, with the periplasm- and cytoplasm-facing surfaces toward the top and bottom, respectively. (B)

Binding sites for GSSG illustrated in the same orientation as (A), with the ligand depicted as space-filling models; primary and secondary binding sites are represented by green and yellow carbons, respectively. (C) Representation of one NaAtm1 subunit emphasizing the secondary structure arrangement, based on the Sav1866 nomenclature (22). ICL, intracellular loop.

(TM) helices fused to the nucleotide-binding domain (NBD) (Fig. 1). *NaAtm1* adopts an inward-facing conformation that most closely resembles (fig. S2) the structures of a *Thermotoga maritima* ABC transporter (24) and the human ABCB10 transporter (26).

Because functional studies for *NaAtm1* have not been reported, we implemented three approaches to identify potential substrates for transport: (i) the ability of ligands to stimulate adenosine triphosphatase (ATPase) activity (16, 28, 29); (ii) in vitro transport assays measuring uptake into *E. coli* membrane vesicles; and (iii) in vivo susceptibility assays to assess the ability of *NaAtm1* to restore tolerance to heavy metals in sensitive *E. coli* strains (30, 31). As observed for *S. cerevisiae* Atm1p (16), the ATPase activity of *NaAtm1* was stimulated by GSH and related compounds, with the highest activity (defined by the ratio of the catalytic rate constant k_{cat} to the Michaelis constant K_m) observed for metallated, aromatic hydrocarbon-conjugated, and oxidized GSH derivatives (Table 1 and figs. S3 and S4). For the most active substrates, the ATPase activity was stimulated by a factor of 3 to 7 over the basal rate. Effective stimulation of ATPase activity by ligands appears to require both free α -carboxyl and α -amino groups, because glycine and the γ -peptides GSH, γ -Glu-Cys, and ophthalmic acid (glutathione with the thiol group replaced with methyl) exhibited stronger ATPase activities relative to Cys-Gly and monocarboxylic acids (Table 1); these properties contrast with yeast Atm1, in which the GSH thiol group is critical for efficient ATPase activity (16). However, the presence of α -amino and carboxyl groups in the transported ligand, although apparently necessary, is not sufficient for ATPase activity, as evidenced by the low activity of many amino acids (fig. S5). Further demonstration of the substrate specificity of *NaAtm1* was provided by direct transport assays, where we observed ATP-driven, *NaAtm1*-dependent uptake of oxidized glutathione (GSSG) into *E. coli* membrane vesicles (Fig. 2A). The ability of *NaAtm1* to transport GSSG distinguishes it from the *E. coli* CydDC ABC transporter that is selective for reduced glutathione (32).

The greatest stimulation of ATP hydrolysis by *NaAtm1* was observed for the Ag and Hg complexes of GSH (Table 1). In view of the toxicity of these metals at low intracellular concentrations, we tested the in vivo relevance of the ATPase results by means of metal susceptibility assays (30, 31). Consistent with a role for *NaAtm1* in exporting Ag-GSH and/or Hg-GSH, expression of *NaAtm1* in metal-sensitive *E. coli* strains conferred protection against otherwise toxic concentrations of Ag^+ and Hg^{2+} (Fig. 2, B and C). Taken together, these results suggest that *NaAtm1* mediates resistance to heavy metal toxicity, likely through export of metallated GSH complexes, although other functional properties probably remain to be identified.

As illustrated with GSSG, the major binding site is located ~ 5 Å into the transmembrane region, dominated by interactions from helices TM5 and TM6 and accessible from the cytoplasmic side (Fig. 3, A and B, and fig. S6). This location is ~ 15 Å closer to the cytoplasmic surface than the ligand-binding sites in the mouse P-glycoprotein structure (23, 33), but the majority of those interactions also involve TM5

and TM6 as well as the dimeric equivalent TM11–12. Key interactions are formed between the free amino and carboxyl groups of GSSG and the protein. The α -carboxyl group of the γ -Glu forms hydrogen bonds with the amide NHs of Gly³¹⁹ and Met³²⁰ in TM6 and with the side chain of Gln²⁷² (TM5), while the α -amino group of Glu interacts with the peptide carbonyl of Asp³¹⁶ (TM6) and the side chains of Asn²⁶⁹ and Gln²⁷² (TM5). At the other end of the glutathione tripeptide, the α -carboxylate of the Gly forms a hydrogen bond to the side chain of Tyr¹⁵⁶ (TM3) on the opposing subunit from the one interacting with the γ -Glu residue of GSH (fig. S7). The residues on TM3 and TM6 interacting with GSSG are positioned near helical irregularities, including a stretch of 3_{10} helix between residues 314 and 317 following a kink at the conserved Pro³¹⁴ in TM6, and a π helix between residues 158 and 162 in TM3; these may represent regions that undergo conformational changes during the transport cycle (34). The nonpolar contacts between the protein and ligand are primarily formed by the side chains of the broadly conserved residues Leu²⁶⁵ and Leu²⁶⁸ packing against

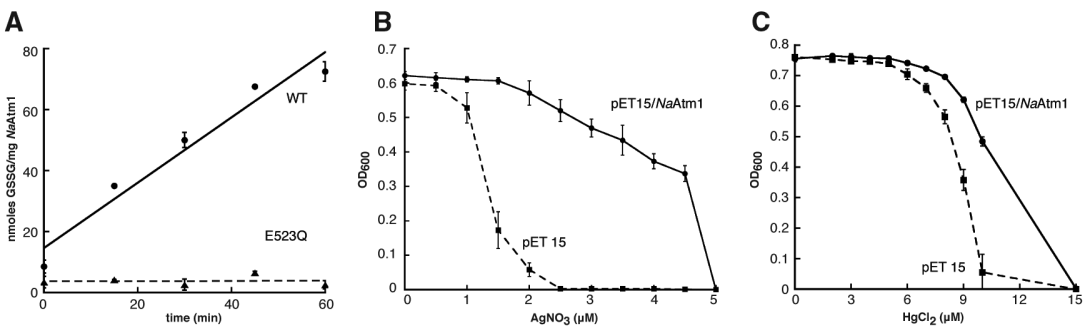
Table 1. Kinetic constants for the ATPase activity of selected substrates derived from a Michaelis-Menten type analysis. The measured ATPase activity is fit to the equation $v/[E]_T = k_{cat}[S]/(K_m + [S]) + k_{basal}$, where v is the measured ATPase activity, $[E]_T$ is the total concentration of *NaAtm1*, $[S]$ is the substrate concentration, and k_{basal} is the basal rate of ATPase activity, which is measured to be $8.8 \pm 0.8 \text{ min}^{-1}$ in 1 mM ATP.

Substrate	$k_{cat}/K_m \text{ (min}^{-1} \text{ mM}^{-1}\text{)}$	$K_m \text{ (mM)}$	$k_{cat} \text{ (min}^{-1}\text{)}$
S-Ag GSH*	2400	0.012 ± 0.001	29 ± 1
S-Hg GSH*	200	0.12 ± 0.04	24 ± 1
S-Dinitrobenzene GSH	120	0.21 ± 0.05	26 ± 2
S-Hexyl GSH	100	0.40 ± 0.12	40 ± 4
S-Bimane GSH	47	1.2 ± 0.2	56 ± 4
GSSG	29	0.97 ± 0.12	28 ± 1
GSH	3.8	15 ± 1	57 ± 2
Glycine	2.8	17 ± 3	47 ± 4
γ -Glu-Cys	2.6	10 ± 2	26 ± 2
Ophthalmic acid	1.7	36 ± 5	62 ± 6
Cys-Gly	0.13	46 ± 35	6.2 ± 3.2
Sodium acetate	0.13	10 ± 21	1.3 ± 1.2

* K_m is reported as per mole of Ag^+/Hg^{2+} basis.

Fig. 2. In vitro and in vivo assays of *NaAtm1* function.

(A) Amount of GSSG transported per milligram of protein accumulated inside vesicles prepared from *E. coli* membranes over-expressing wild-type *NaAtm1* or the E523Q (Glu⁵²³ → Gln) Walker B motif ATPase-defective mutant. (B) Optical density of cells after 12 hours of growth in the presence of the indicated $AgNO_3$ concentrations. *E. coli* strain GG44 (Cu^+/Ag^+ sensitive) was transformed with either an empty pET15 plasmid or a pET15 plasmid encoding *NaAtm1*. (C) Optical density of cells after 12 hours of growth in the presence of the indicated $HgCl_2$ concentrations. *E. coli* strain GG48 ($Zn^{2+}/Cd^{2+}/Hg^{2+}$ sensitive) was transformed as in (B). Error bars represent SD of three independent measurements.



the Gly end of GSSG, and by the more variable side chains of Met³¹⁷ and Met³²⁰ interacting near the disulfide of GSSG (fig. S8).

A second, lower-occupancy, binding site for GSSG is positioned ~5 Å from the primary site toward the cytoplasmic surface. The relative positions of the two GSSG sites are suggestive of a potential binding mode for a [2Fe:2S](GS)₄ complex (35), although the actual physiological relevance of this site is not known. The binding site involves conserved arginine residues 206 and 323, along with the more variable Arg²¹⁰ (Figs. 1B and 3B). Residue Thr³²⁴ is the counterpart of residue Glu⁴³³ in human ABCB7 that is mutated to Lys in X-linked sideroblastic anemia (7). Two additional pairs of Arg residues are positioned in the translocation pathway toward the periplasmic side [the conserved Arg⁹¹ and the more variable Arg³¹³ (fig. S9)] that perhaps represent a transient binding site for ligands in the outward-facing conformation.

The functional importance of these interactions was assessed by evaluating the consequences of site-directed mutagenesis and substrate variation on the ATPase activity. Residues Asn²⁶⁹, Gln²⁷², and Gly³¹⁹ that form hydrogen bonds to the α -amino and carboxylate groups of the γ -Glu are highly conserved, and substitution of these residues alters the ATPase activity (Fig. 3, C and D). Although mutations of these residues (Asn²⁶⁹ → Ala, Gln²⁷² → Ala, Gly³¹⁹ → Leu) result in varying degrees of ATPase activity, they all effectively minimize GSH stimulation. The Asn²⁶⁹ → Ala substitution both enhances the basal ATPase activity and eliminates GSH sensitivity, whereas incorporation of a bulky residue at position 319 (Gly → Leu) or Gln²⁷² → Ala mutation substantially reduces both the GSH sensitivity and the basal ATPase activity. The minimal ATPase activity of the Gln²⁷² → Ala variant, together with the ability of the free amino acids Gly and Met to stimulate ATPase

activity as effectively as GSH, suggests that the binding of ligands with α -amino and carboxyl groups to the Gln²⁷² site in TM5 can trigger the conformational changes associated with ATP hydrolysis (Fig. 3C and fig. S10). As a potential coupling mechanism, Gln²⁷² is adjacent to the side chain of Tyr¹⁹⁵ in TM4 that forms a hydrogen bond with the backbone carbonyl of Leu³¹⁵ in TM6, thereby linking TM4 and TM5 to TM6 and the following NBD. Substitution of these residues yields interesting phenotypes (Fig. 3D); the Tyr¹⁹⁵ → Phe variant exhibits substrate-insensitive ATPase hyperactivity, which suggests that the native Tyr¹⁹⁵ contact to TM6 may stabilize the inward-facing conformation. Conversely, because the Gln²⁷² → Ala variant is essentially ATPase-inactive, interactions of this residue with the binding of an amino acid motif may be required to unlock the protein from the cytoplasm-facing conformation (Fig. 3C). The double mutant Tyr¹⁹⁵ → Phe/Gln²⁷² → Ala, however, has a high basal ATPase activity without GSH stimulation, indicating that when neither residue can potentially interact with TM6, the transporter can facilitate undergo the conformational changes associated with ATPase activity.

The interconversion between inward- and outward-facing conformations of ABC exporters coupled to ATP hydrolysis is generally described within the framework of the alternating access transport mechanism (36). However, an analysis of MsbA structures solved in multiple conformational states emphasized that this interconversion cannot be described in terms of rigid body movements of subunits, but rather rearrangements that occur at the interfaces between certain TM helices (27). More specifically, the alternating conformations of ABC exporters may be distinguished by the separation of either TM1 and TM2 (outward) or TM4 and TM5 (inward) from the remaining four TM helices within the same subunit (22, 27). Examination of the available structures of ABC exporters identifies four elements that are generally conserved structurally: TM1–2, TM4–5, and TM3&6 (Fig. 4A), together with the NBD, that is the hallmark of this family. These pairs of TM helices reflect the approximate symmetry in the transmembrane domain (TMD) noted in the Sav1866 structure (22), where TM1–3 and TM4–6 are related by a two-fold axis between TM3 and TM6 perpendicular to the molecular two-fold axis. The conformational changes associated with transport involve rearrangements in the relative orientations of these conserved elements (Fig. 4B), mediated by their interactions with the ligand and ultimately coupled to the NBDs through intracellular loop 1 (ICL1) between TM1 and TM2, intracellular loop 2 (ICL2) between TM4 and TM5, and the covalent connection from TM6 to the NBDs (Fig. 1). ABC exporters can be considered to have an articulated construction; as this work demonstrates, transported ligands span these structurally conserved elements in the TMD to create adaptable interfaces

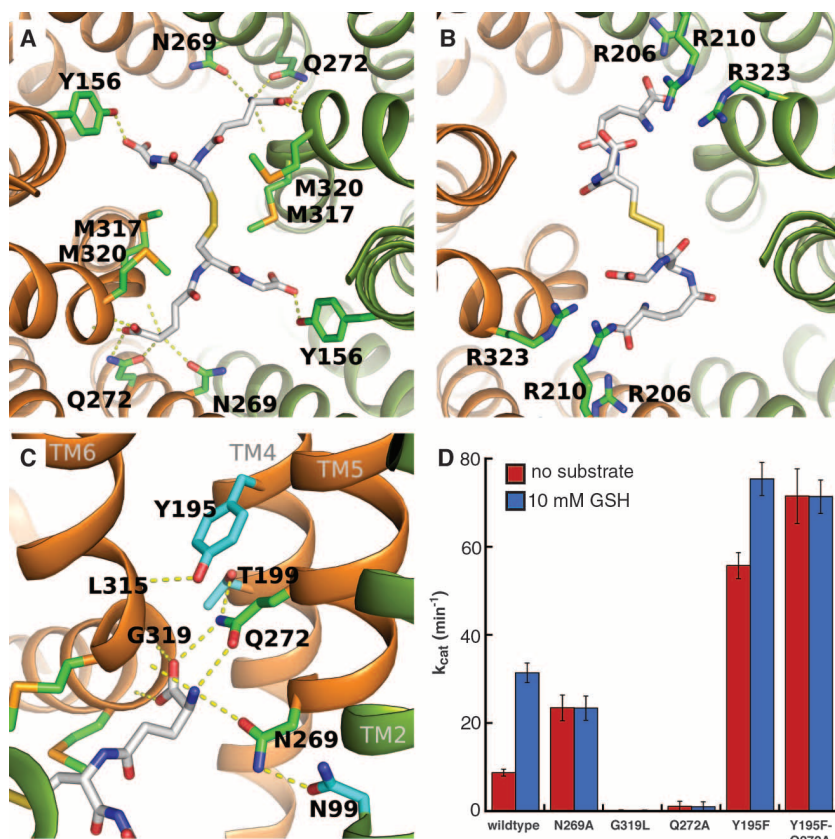
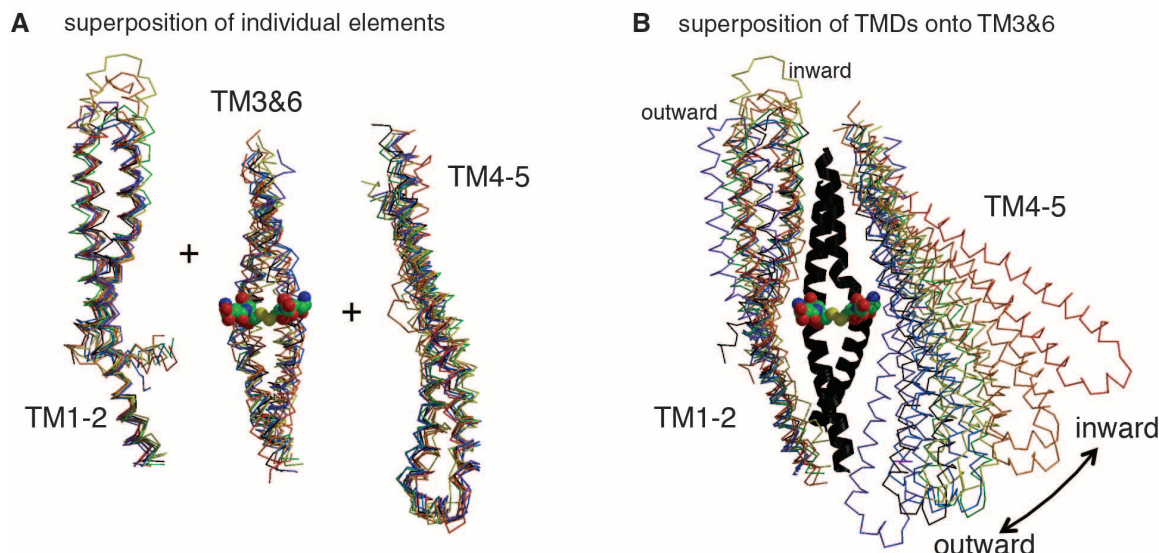


Fig. 3. Binding sites of glutathione derivatives to NaAtm1. (A) The primary binding site of GSSG, with the substrate and interacting side chains depicted as bonds and the polypeptide backbone in ribbons. The substrate carbons are colored light gray; the protein side-chain carbons are green. Hydrogen bonds between the protein and ligands are represented by yellow dashes. (B) The secondary binding site for GSSG. (C) Interactions between the γ -Glu of GSH and surrounding residues of NaAtm1. The carbon atoms of GSH are shaded light gray; green carbons denote side chains directly interacting with GSH, and blue carbons indicate side chains interacting with (green) residues interacting with GSH. (D) ATPase activity (k_{cat}) of selected mutations in the ligand-binding site; rate constants in the absence of substrate and in the presence of 10 mM GSH are shown. Error bars represent SD of three independent measurements. Amino acid abbreviations: A, Ala; F, Phe; G, Gly; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; T, Thr; Y, Tyr.

Fig. 4. Structural conservation and the articulated construction of ABC exporters. (A) Structural conservation of the individual TM1–2, TM3&6, and TM4–5 elements in the TMDs of ABC exporter structures. The *NaAtm1* structure was used for the reference, with the TM1–2 and TM4–5 superpositions horizontally displaced from TM3&6 by 20 Å to the left and right, respectively. The GSSG ligand in the primary binding site is depicted by a space-filling model with the carbons colored green. The underlying symmetry in the TMD, first noted in the Sav1866 structure (22), relates TM1–3 and TM4–6 by a two-fold rotation axis passing between TM3 and TM6 and normal to the plane of the page. (B) Variations in the relative orientations of conserved TMD elements observed in the structures of ABC exporters. The TMDs of different exporters were aligned with *NaAtm1* using only TM3&6 for the superposition; for clarity, only TM3&6 from *NaAtm1*, depicted with black ribbons, is shown. Although the individual TM1–2 and TM4–5 elements are structurally conserved as shown in (A), substantial changes in their relative positions are evident between these structures (espe-



accommodating conformational rearrangements during the transport cycle. Mutations in residues implicated in mediating such contacts have been identified in cystic fibrosis transmembrane conductance regulator (CFTR) variants, including residues Arg³⁴⁷ and Asp⁹⁹³ (37, 38) in TM6 and TM9, respectively. These residues spatially correspond to positions 316 and 157 in TM6 and TM3 on different subunits of *NaAtm1* [based on the TM3&6 structural alignment of *NaAtm1* with the Sav1866-based homology model of CFTR (39)], near the GSH binding site. This suggests that the interactions observed here are of broader relevance for influencing the conformational equilibria essential for proper functioning of ABC exporters.

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Supplementary Materials

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Figures S1 to S10
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Crystal Structures of Nucleotide-Free and Glutathione-Bound Mitochondrial ABC Transporter Atm1

Vasundara Srinivasan *et al.*

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Crystal Structures of Nucleotide-Free and Glutathione-Bound Mitochondrial ABC Transporter Atm1

Vasundara Srinivasan,^{1,2*} Antonio J. Pierik,^{1†} Roland Lill^{1,2,3*}

The yeast mitochondrial ABC transporter Atm1, in concert with glutathione, functions in the export of a substrate required for cytosolic-nuclear iron-sulfur protein biogenesis and cellular iron regulation. Defects in the human ortholog ABCB7 cause the sideroblastic anemia XLSA/A. Here, we report the crystal structures of free and glutathione-bound Atm1 in inward-facing, open conformations at 3.06- and 3.38-angstrom resolution, respectively. The glutathione binding site includes a residue mutated in XLSA/A and is located close to the inner membrane surface in a large cavity. The two nucleotide-free adenosine 5'-triphosphate binding domains do not interact yet are kept in close vicinity through tight interaction of the two C-terminal α -helices of the Atm1 dimer. The resulting protein stabilization may be a common structural feature of all ABC exporters.

Mitochondria contain several adenosine 5'-triphosphate (ATP) binding cassette (ABC) transporters that are crucial for organellar communication with the cytosol and nucleus (1–4). These proteins are located in the mitochondrial inner membrane with the nucleotide binding domain (NBD) facing the matrix and hence are thought to function as exporters. Yeast Atm1, vertebrate ABCB7, and plant Atm3 are involved in the biogenesis of cytosolic-nuclear iron-sulfur (Fe/S) proteins, including DNA polymerases, primases, and helicases involved in

genome integrity (5–14). The sulfur present in cytosolic-nuclear Fe/S proteins is generated from cysteine inside mitochondria by the cysteine desulfurase complex Nfs1-Isd11. This complex and other members of the mitochondrial iron-sulfur cluster (ISC) assembly machinery synthesize an unknown, sulfur-containing molecule that is exported by Atm1 and used by the cytosolic Fe/S protein assembly (CIA) system (15, 16). This molecule also signals the iron status to the nucleus (17). Upon ISC protein or Atm1 depletion, cellular iron uptake is increased, and mitochondria accumulate up to 30-fold higher iron concentrations (5, 9, 18, 19). Depletion of the sulfhydryl oxidase Erv1 or the redox molecule glutathione (GSH) shows a strikingly similar phenotype to Atm1 deficiency, suggesting that the three components cooperate in the export process (20–23). In fact, Atm1-deficient mitochondria show an increased content of GSH (18). Moreover, the adenosine triphosphatase (ATPase) activity of Atm1 is stimulated by GSH, suggesting that this tripeptide may be directly involved in the export process (24).

Homologs of yeast Atm1 are found in virtually all eukaryotes and some bacteria and usually show ~50% sequence identity (fig. S1A). The function of the bacterial Atm1-like proteins is unknown, but they may also participate in the biogenesis of a metal-sulfur center (25). The human, mouse, and plant proteins can replace yeast Atm1, underlining the conserved function of this ABC transporter in eukaryotic Fe/S protein biogenesis and iron regulation. Mutations in human ABCB7 cause X-linked sideroblastic anemia and cerebellar ataxia (XLSA/A), an iron storage disease characterized by diminished cytosolic Fe/S protein function, iron-loaded mitochondria (sideroblasts), and defects in heme metabolism (26–29). Recently, decreased expression of ABCB7 was claimed to play a role in refractory anemia with ring sideroblasts (RARS), a myeloid malignancy that can transform to acute leukemia (30, 31).

Structural information on Atm1 is essential for better understanding of its central function in cellular iron metabolism, identification of its substrate, and elucidation of the transport mechanism. To date, structural information on eukaryotic ABC transporters is available only for P-glycoprotein (P-gp), which is implicated in multidrug resistance (MDR), and the mitochondrial ABCB10, which is involved in heme metabolism in erythroid cells (32–34); yet, these proteins differ from Atm1 in tissue specificity, intracellular localization, subunit composition, function, and substrate. We solved the three-dimensional structure of *Saccharomyces cerevisiae* Atm1 with x-ray crystallography using the single anomalous dispersion (SAD) method (Fig. 1 and fig. S1B) (35). The experimental phases were obtained from soaking the native crystals with tantalum bromide cluster (Ta₆Br₁₂) (fig. S2). Data collection and refinement statistics are summarized in table S1. A stereoview of the (2Fo-Fc) electron density map for the complete Atm1 dimer is shown in fig. S3. The final model encompasses the complete polypeptide sequence of Atm1

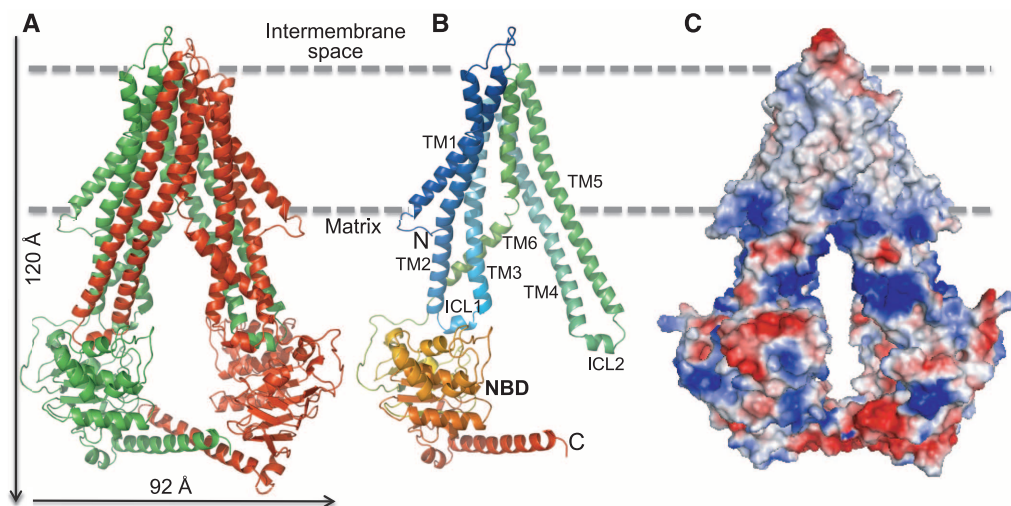
¹Institut für Zytobiologie, Philipps-Universität Marburg, Robert-Koch-Strasse 6, 35032 Marburg, Germany. ²LOEWE Zentrum für Synthetische Mikrobiologie SynMikro, Hans-Meerwein-Strasse, 35043 Marburg, Germany. ³Max-Planck-Institut für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse 10, 35043 Marburg, Germany.

*Corresponding author. E-mail: lill@staff.uni-marburg.de (R.L.); vasundara.srinivasan@staff.uni-marburg.de (S.V.)

†Present address: Fachbereich Chemie, Technische Universität Kaiserslautern, Erwin-Schrödinger-Strasse 54, 67663 Kaiserslautern, Germany.

Fig. 1. Crystal structure of the mitochondrial ABC transporter Atm1.

(A) Illustration of the nucleotide-free Atm1 homodimer (*S. cerevisiae*), with monomers colored in green and red and the NBDs facing the mitochondrial matrix. The approximate positioning of the lipid bilayer is indicated by dashed lines. (B) The Atm1 monomer depicted in rainbow colors shows the crossover of the TM4 and TM5 helices to the other monomer (not shown), resulting in the formation of a V-shaped molecule with a large cavity. The N and C termini, the NBDs, and the coupling helices ICL1 and ICL2 that form the transmission interface between the transmembrane helices and the NBDs are indicated. (C) Electrostatic surface potential representation of the Atm1 dimer (blue, positive; red, negative; and white, neutral). The substrate binding cavity located near the hydrophilic surface of the membrane is positively charged.



except for the first 98, nonessential residues, including the mitochondrial presequence (fig. S1A) (24). Two nucleotide-free structures were determined, one without and one with bound GSH to a resolution of 3.06 and 3.38 Å, respectively (Figs. 1 and 2A). Both structures were captured in an inward-facing, open conformation that conforms to the known eukaryotic ABC exporter fold and represents a pretranslocation state. The GSH-bound and -free structures were virtually identical, with an overall root-mean square deviation (RMSD) of 0.3 Å for the complete molecule (fig. S4).

Atm1 assembled as a dimer in the crystallographic lattice (fig. S5), and the molecules were packed in a “head to tail” arrangement. The ATPase activity of Atm1 was almost fully recovered after detergent solubilization of the crystals, suggesting that purification and crystallization did not affect function (fig. S6). The dimer consists of 12 transmembrane (TM) helices in two bundles of six helices per monomer. The six TM helices from each monomer do not form a single structural unit; rather, the long TM4 and TM5 helices reach out to the other monomer,

thus forming an intertwined “domain-swapped” membrane-integrated arrangement (Fig. 1, A and B). This configuration creates a V-shaped molecule enclosing a 6900 Å³, positively charged cavity close to the hydrophilic phase of the matrix side of the inner membrane (Fig. 1C).

In several crystals, this cavity contained an electron density that fits best to GSH (Fig. 2). The ligand binding region corresponding to TM4, TM5, and TM6 was well resolved (fig. S7). Both purified Atm1 and the crystals contained GSH as verified by means of mass spectrometry, biochemical analysis, and equilibrium titration [apparent dissociation constant (K_d) = 23 μM] monitored with microscale thermophoresis (figs. S8 and S9A) (36). Verapamil, a molecule transported by the multidrug transporter P-gp, did not bind to Atm1 (fig. S9B). Because both purification and crystallization were performed without GSH, the compound must have associated with Atm1 after its synthesis in *Escherichia coli*, indicating stable complex formation during purification at low temperature. A bacterial homolog of Atm1 binds GSH in a similar location (37). The binding site of the Atm1-bound GSH ligand substantially differed from that of hydrophobic inhibitors of P-gp (32), despite the similar size and shape of the cavities of both transporters (fig. S10). These findings are consistent with the anticipated entry points of hydrophilic ligands such as GSH, as opposed to hydrophobic P-gp substrates that are thought to reach the cavity from the lipid phase (32). Last, GSH docking calculations with the Atm1 crystal structure coordinates supported a high-affinity binding site for GSH within the cavity, in close vicinity to the GSH binding site observed in the crystal structure (fig. S11).

The cavity in the Atm1 structure is lined with mostly charged and polar amino acid residues

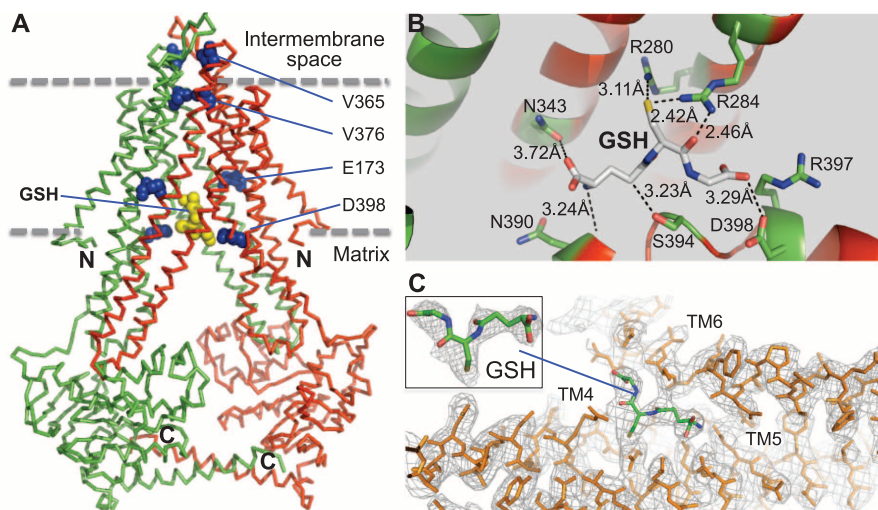
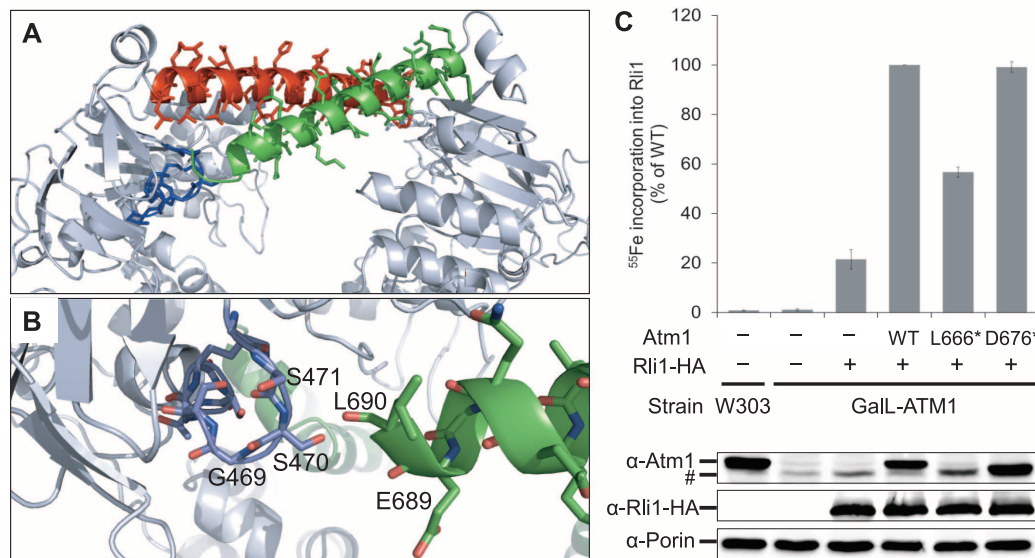


Fig. 2. Crystal structure of mitochondrial Atm1 in complex with glutathione. (A) GSH represented by yellow spheres is well accommodated in the cavity formed by the Atm1 dimer. The residues corresponding to those mutated in XLSA/A disease are marked as blue spheres. (B) GSH (shown in stick representation) mainly interacts by forming hydrogen bonds with residues D398, R284, and R280, indicated by black dashed lines. The other residues that line the cavity are N390, N343, S394, and R397. (C) A section of the experimental (2Fo-Fc) electron density map contoured at 1σ (gray) shows Atm1-bound GSH (stick representation) in close vicinity to TM4, TM5, and TM6 (orange sticks). (Inset) A close-up view of the electron density for GSH from the same map.

Fig. 3. The C-terminal α-helices stabilize the Atm1 dimer in the inward-facing, open conformation.

(A) The Atm1 NBDs are presented in gray, the Walker A loop in blue, and the two crossover C-terminal helices (table S2) in green and red as an illustration (side chains as sticks). The C-terminal helices include five residues of the strep-tag used for purification (side chains not shown for clarity). (B) The C-terminal residues E689 to L690 of one Atm1 monomer (green) almost touch the Walker A loop (G469 to S471, sticks) of the other monomer (blue). Residues of the strep tag were omitted here. (C) Wild-type (W303) or GalL-ATM1 yeast cells were transformed with plasmids encoding no protein (–), the model Fe/S protein Rli1-HA, and wild-type (WT) and mutated Atm1 (asterisk, truncated). Cells were grown for 64 hours in SD medium for depletion of Atm1, radiolabeled with ⁵⁵Fe, and analyzed for ⁵⁵Fe/S cluster insertion into Rli1-HA with immunoprecipitation and scintillation counting. (Bottom) Indicated proteins were visualized by the immunoblotting of cell extracts (pound sign, cross-reacting band).



and likely serves as the substrate binding site. The two TM6 helices bend out of the membrane, forming a “kink” at residue S394 before connecting to the NBD through the linker region, offering flexibility to accommodate the GSH ligand (Fig. 2B). (Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.) GSH fits snugly to a positively charged cavity through interaction with residues R280 and R284 (TM4), N343 (TM5), N390, S394, R397, and D398 (TM6) (Fig. 2, B and C, and fig. S7). All GSH-coordinating residues are conserved in Atm1-like proteins, including human ABCB7 and bacterial homologs (fig. S1A). One of these residues (D398) corresponds to human E433, which is changed to lysine in patients with XLSA/A, clearly showing the physiological relevance of GSH ligand binding (Fig. 2A) (27). Both yeast Atm1 and human ABCB7 proteins carrying this mutation show diminished activity in the maturation of cytosolic Fe/S proteins. Likely, the site of GSH interaction is part of the substrate binding region of Atm1. GSH itself cannot be the physiological substrate because it is synthesized in the cytosol. What may be the identity of the Atm1 substrate? Cell biological data demonstrate that Atm1 exports a sulfur-containing molecule generated by the core mitochondrial ISC assembly machinery (15, 16). GSH may be part of the exported moiety because GSH, like Atm1, is essential for both cytosolic-nuclear Fe/S protein biogenesis and cellular iron regulation (21–23). Therefore, in the simplest view GSH may be converted to glutathione persulfide (GSSH) and directly used as a sulfur carrier. Alternatively, a GSH-coordinated [2Fe-2S] cluster was reported to be stable in water (38). This or a similar compound may be synthesized by the mitochondrial ISC system and exported by Atm1. Atm1 structures determined from crystals soaked or cocrystallized with such compounds will help identifying its substrate.

Three of the XLSA/A patient mutations are located in the membrane domain either on the matrix [E208D in long TM2 helix; yeast E173 (29)] or intermembrane space sides [I400M between TM5 and TM6, yeast V365 (26); V411L in TM6, yeast V376 (28)] (Fig. 2A and fig. S1A). (In the mutants, other amino acids were substituted at certain locations; for example, E208D indicates that glutamic acid at position 208 was replaced by aspartic acid.) All of these amino acid exchanges are conservative (fig. S1A), indicating that even subtle changes in ABCB7 function may lead to the disease phenotype. Conspicuously, V376 in yeast Atm1 undergoes hydrophobic interactions with its counterpart residue in the other monomer, thus tightly closing the transmembrane channel toward the intermembrane space (Fig. 2A and fig. S12). Replacement of valine by the larger hydrophobic residue leucine is pathogenic (28). The mutation likely

affects the tightness of the transport channel and/or the mobility of the transmembrane segments during the transport process.

One key feature of both Atm1 structures was the complete resolution of the C terminus that consists of an α -helix 24 amino acid residues long (Fig. 3A and fig. S1A). Such a structural feature has not been observed previously in any other ABC transporter crystal structure, although these proteins usually contain C-terminal segments that potentially could form α -helices. A representative electron density map (2Fo-Fc) contoured at 1 σ for the C-terminal helix reveals an unambiguous density in this region (fig. S13). The helix is in close proximity to its counterpart helix from the second monomer, and both helices tightly interact in a crossover fashion, with distances ranging from 2.65 to 4.0 Å (Fig. 3B and table S2). The two helices form a connecting bridge between the two NBDs, thus locking the Atm1 dimer in the inward-facing, open conformation. Further, the α -helix from one monomer is long enough to make extensive contacts to residues G469 to S471 of the Walker A motif of the other monomer (Fig. 3B). It is evident that during the ABC transporter working cycle, the two α -helices either have to unlock and slide along each other or become more tilted to allow the tight interaction of the two NBDs after ATP binding. A superposition of the NBD structure of Atm1 with those of the known ABC exporters (mouse and *Caenorhabditis elegans* P-gp, bacterial multidrug-resistance protein Sav1866, and human mitochondrial ABCB10) clearly reveals the unprecedented character of the C-terminal α -helix of Atm1 (fig. S14). The short helix seen in *C. elegans* P-gp is present at the end of only one of the NBDs.

Flexible interactions at the C termini might be a common feature of many if not all ABC exporters and may serve to stabilize the protein. To test this idea, we truncated the C-terminal part of Atm1 to remove half or all of the helix (mutants D676* and L666*, respectively). The mutant proteins were expressed from plasmids in regulatable GalL-ATM1 yeast cells in which the endogenous Atm1 can be depleted by growth in the presence of glucose (5). Both mutant proteins rescued the growth defect of Atm1-depleted cells at 30°C; yet at 37°C, a slightly slower growth was observed for Atm1-L666* (full deletion of α -helix) (fig. S15A). The growth defect was accompanied by substantially diminished protein levels of Atm1-L666* and a 50% reduced activity in the biogenesis of a representative cytosolic Fe/S protein Rli1 (Fig. 3C) (39). A similar diminution in the extent of Fe/S protein assembly can cause XLSA/A (27). Nevertheless, the increased iron levels of Atm1-depleted mitochondria could be normalized by both Atm1 mutant proteins (fig. S15B), suggesting that the truncation mainly affected protein stability rather than function.

On the basis of the two structures reported here, a model for substrate translocation is pro-

posed. The Atm1 substrate enters the inward-facing, open structure from the matrix side to bind in the hydrophilic cavity (fig. S16). Because the GSH ligand was bound in the absence of ATP, substrate binding may occur before nucleotide binding. The scissor-like interaction of the two C-terminal α -helices of the Atm1 dimer may keep the two NBDs close together before their ATP-induced, tight association, stabilizing the protein. This effect may be relevant for other bacterial and eukaryotic ABC exporters, at least under adverse environmental conditions. The ATP-driven structural rearrangement leads to an outward-facing conformation that may be similar to a homology model calculated for Atm1 (fig. S16, right) by using a structure of the Sav1866 ABC transporter (40). As a result of the intimate NBD contact, the transmembrane helices rearrange and thereby create an exit pathway for substrate release. The pretranslocation state can be understood on the basis of the crystal structures, yet the complex ATP-driven alterations require further studies. Our structures will boost further mechanistic insights into Atm1-ABCB7 function, substrate interaction, and pathology.

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Supplementary Materials

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References (41–61).

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Mechanism of the C5 Stereo-inversion Reaction in the Biosynthesis of Carbapenem Antibiotics

Wei-chen Chang,^{1*} Yisong Guo,^{1†} Chen Wang,² Susan E. Butch,¹ Amy C. Rosenzweig,³ Amie K. Boal,^{1,2,3*} Carsten Krebs,^{1,2*} J. Martin Bollinger Jr.^{1,2*}

The bicyclic β -lactam/2-pyrrolidine precursor to all carbapenem antibiotics is biosynthesized by attachment of a carboxymethylene unit to C5 of L-proline followed by β -lactam ring closure. Carbapenem synthase (CarC), an Fe(II) and 2-(oxo)glutarate (Fe/2OG)-dependent oxygenase, then inverts the C5 configuration. Here we report the structure of CarC in complex with its substrate and biophysical dissection of its reaction to reveal the stereo-inversion mechanism. An Fe(IV)-oxo intermediate abstracts the hydrogen (H•) from C5, and tyrosine 165, a residue not visualized in the published structures of CarC lacking bound substrate, donates H• to the opposite face of the resultant radical. The reaction oxidizes the Fe(II) cofactor to Fe(III), limiting wild-type CarC to one turnover, but substitution of the H•-donating tyrosine disables stereo-inversion and confers to CarC the capacity for catalytic substrate oxidation.

β -Lactam compounds constitute more than half of the global antibiotic drug market (1, 2). In the past decade, the increased incidence of bacterial β -lactam resistance has forced increasing reliance on a relatively new subclass of these drugs known as carbapenems (Fig. 1A) (2, 3). Defined by the bicyclic β -lactam/pyrrolidine nucleus (1), carbapenems are generally less susceptible than many members of the β -lactam class to hydrolytic inactivation by β -lactamases. With more than 40 naturally occurring members, the carbapenem subclass retains activity against a broad range of both Gram-positive and Gram-negative bacteria, including *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (4). An understanding of the biosynthetic pathways to these natural carbapenems could enable the engineering of new drug variants to combat resistance, which has been emerging at an alarming rate, even to this most robust β -lactam subclass (5).

After the initial isolation of a carbapenem, thienamycin (2), from *Streptomyces cattleya* in

1976 (6), the structurally simplest carbapenem, **1**, was isolated from *Erwinia carotovora* (known also as *Pectobacterium carotovorum*) and other species (7). Early biochemical studies identified the *car* gene cluster encoding the enzymatic apparatus to produce **1** (8, 9). Later studies confirmed that this cluster is conserved in all strains that produce carbapenem and is distinct from gene clusters specifying penicillin and cephalosporin biosynthesis (2). Introduction of the *carA*, *carB*, and *carC* genes into *Escherichia coli* established that the three encoded enzymes, carbapenam synthetase (CarA), carboxymethylproline synthase (CarB), and carbapenem synthase (CarC), are sufficient to produce **3**, **4**, and **1** and enabled in vitro dissection of the biosynthetic sequence (10) (Fig. 1A). Using malonyl-coenzyme A (CoA) as co-substrate, CarB attaches a carboxymethylene unit to 1-pyrroline-5-carboxylate (which may be supplied in carbapenem-producing microorganisms from L-proline by the *carD* gene product) to form *trans*-5-carboxymethyl-L-proline (11, 12). CarA then uses adenosine 5'-triphosphate hydrolysis (to adenosine 5'-monophosphate and pyrophosphate) to drive β -lactam ring closure, generating (3*S*,5*S*)-carbapenam, **3** (13). The stereochemical configuration at C5 established by CarB is opposite to that in all natural carbapenem antibiotics isolated to date (2). Further elaboration of **3** apparently requires C5 stereo-inversion by CarC, an Fe(II) and 2-(oxo)glutarate (Fe/2OG)-dependent oxygenase, which produces (3*S*,5*R*)-carbapenam, **4**, as its initial product (10). It has been concluded

that only after inversion of C5 can CarC then de-saturate across the C2-C3 bond to produce (5*R*)-carbapenam, **1** (14). The *R* configuration at C5 and the C2=C3 double bond of **1** are both crucial for optimal antibiotic activity (15, 16). To our knowledge, no CarB ortholog has yet been shown to produce the *cis*-5-carboxymethyl-L-proline, with C5 in the configuration found in the drug compounds (2, 17–19). Moreover, although it has been suggested that C5 epimerases structurally and mechanistically unrelated to CarC might be operant in other carbapenem biosynthetic pathways [e.g., to thienamycin (17, 20)], none has been identified to date. An understanding of the structure and function of CarC is thus central to efforts to bioengineer new carbapenems.

Mononuclear non-heme-iron oxidases and oxygenases, including those in the Fe/2OG subclass, generally effect oxidation of their primary substrates (21). In the most well-understood cases, addition of O₂ to the Fe(II) cofactor and delivery of two electrons from a cosubstrate generates an Fe(IV)-oxo (ferryl) intermediate, which either abstracts a hydrogen atom (H•) from an aliphatic carbon of the substrate or adds as an electrophile to a double bond (22). In cases of H• abstraction, an iron ligand then transfers as a radical to the substrate radical, completing the oxidative substitution of hydrogen by a heteroatom (O, Cl/Br, or S; Fig. 1B, reaction i). This step regenerates the Fe(II) form of the cofactor for subsequent turnovers (21–24).

By contrast to these oxidative substitution reactions, the CarC stereo-inversion reaction is redox neutral with respect to its primary substrate, **3**. This outcome has been rationalized by mechanisms invoking H• abstraction from C5 by the canonical ferryl species and subsequent transfer of a different H• to the opposite face of the substrate radical (Fig. 1B, reaction ii) by, presumably, an amino acid donor (X-H in Fig. 1B) (25). Computational analyses have supported such a mechanism and involvement of a dedicated H• donor (26). A fluorescence-based in vivo assay for carbapenem production was coupled with amino acid mutagenesis to obtain data suggesting that tyrosine (Y) 67, visualized near the bound *N*-acetyl-L-proline intended to mimic the substrate in one of two published x-ray crystal structures of CarC, is the H• donor (27, 28). The most recent of these studies used this structure for molecular-docking analysis that identified a hypothetical substrate-binding mode with C5 in sufficient proximity to Y67 for H• transfer

¹Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, USA. ²Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802, USA. ³Department of Molecular Biosciences and Department of Chemistry, Northwestern University, Evanston, IL 60208, USA.

*Corresponding author. E-mail: oscarntu@gmail.com (W.-c.C.); akb20@psu.edu (A.K.B.); ckrebs@psu.edu (C.K.); jmb21@psu.edu (J.M.B.)

†Present address: Department of Chemistry, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA.

(28). However, because multiple segments of the protein are disordered in this structure, its use as a starting point for such an analysis is inherently speculative. Published mechanisms have further suggested that, after inverting C5, CarC then either uses the two oxidizing equivalents still present in the active site (Fig. 1A, pathway ii) or, following their reduction, undergoes a second round of O₂ activation and ferryl formation (pathway i), to effect C2-C3 desaturation (28). As depicted, this second reaction is a mechanistically distinct two-electron oxidation requiring removal of an H• equivalent from each of two adjacent carbon atoms (Fig. 1B, reaction iii) (28, 29).

Studies on other Fe/2OG oxygenases and related oxidative enzymes have established that the crucial tenets of the proposed CarC stereoinversion mechanism, including H• abstraction by a ferryl intermediate and formation of a tyrosyl radical, could be definitively tested by rapid-kinetic and spectroscopic analysis of the reaction (22, 30, 31). In addition, the structure of the enzyme with its authentic substrate, **3**, would be expected to reveal proximity of C5 to the iron cofactor and the H• donor, as intentionally enforced for the case of Y67 in the recent computational study (28). Instability of the substrate (7, 28, 32) and enzyme (28, 33) have repeatedly been cited as barriers to such direct in vitro mechanistic and structural analysis. To obtain the enzyme, we expressed N-terminally His₆-affinity-tagged *Pectobacterium carotovorum* CarC in *E. coli* at 16°C and purified it on Ni(II)-nitrilo-*tris*-acetate agarose affinity resin, obtaining ~30 mg of >90% pure protein per gram of wet cell mass (fig. S1). To obtain the substrate, we made use of the observation by multiple investigators that carboxylate-esterified analogs of **3** are sufficiently stable to be isolated (32, 34, 35). We prepared the methyl ester of **3** (7) and its [⁵-²H]-labeled

isotopolog (5-*d*-7) by a hybrid of two published syntheses (fig. S2A and supplementary methods) and then rapidly, enzymatically hydrolyzed the esters to **3** and 5-*d*-3 just before carrying out in vitro experiments (fig. S2B and supplementary methods). Following this deprotection, we found **3** to have a half-life of ~14 hours at 25°C and pH 7.5 (in 100 mM Tris-chloride).

By this method of in situ deprotection, we overcame the long-standing barrier posed by the substrate's instability to determine both the structure of the CarC•Fe(II)•2OG•**3** quaternary complex and the mechanism of C5 stereoinversion. To enable structural characterization, crystals of the CarC•Fe(II)•2OG complex obtained after a ~24-hour incubation in the absence of O₂ were soaked in freshly prepared anoxic solutions of **3** to form the quaternary complex, and the crystals were frozen before **3** could decompose (after 2 hours; see supplementary methods). The structure, solved at a resolution of 2.1 Å by x-ray diffraction experiments (table S1 and fig. S3), reveals three CarC molecules in the asymmetric unit (ASU). Significant electron density for carbapenam **3** is readily visualized in the active site of two of the three monomers (Fig. 2A and fig. S4), but a crystal lattice contact that distorts the structure of the active site prevents proper substrate binding in the third molecule of the ASU (fig. S5). The bridgehead C5 of **3** resides 4.4 Å from the iron site with its hydrogen directed toward the Fe cofactor in appropriate geometry for its abstraction by the ferryl intermediate (Fig. 2B and fig. S6). This binding orientation is unequivocally established by the shape of the electron density and the positioning of hydrogen-bonding residues to interact with the carboxylate and carbonyl groups of **3**. The presence of the actual substrate [rather than the *N*-acetyl-L-proline bound in one

of two published structures of CarC (36)] yields clear electron density for two previously disordered loops surrounding the active site (fig. S4), allowing their structures to be modeled in two of the three molecules in the ASU. Residues 67 to 78 (loop 1) and 162 to 172 (loop 2) close over the active site, thereby isolating the substrate from bulk solvent. Such protection would seem to be critical for control of the free-radical reaction(s) mediated by CarC, a conclusion that is consistent with the results of recent mutational-saturation studies that showed that substitutions of bulky or aromatic residues in the active site are incompatible with activity (28). In loop 1, Y67 and Y78 adopt new positions to participate with arginine (R) 267 and glutamine (Q) 269 in an extended hydrogen-bonding network that anchors the carboxylate of **3** in the binding pocket. Y164 in loop 2 also interacts directly with the carboxylate. The most mechanistically important change relative to published structures is the ordering of loop 2 to position Y165 directly above C5, opposite the iron center, with its hydroxyl group 4.8 Å from the substrate C5 atom (Fig. 2B). In this position, Y165 is ideally poised to donate H• to the C5 radical to generate the (3*S*,5*R*) epimer, **4**. By contrast, Y67, the residue suggested in previous work to be the H• donor (27, 28), is positioned toward the edge of the substrate, inappropriately to serve in this capacity (Fig. 2 and figs. S4 and S5).

The structure of the CarC•Fe(II)•2OG•**3** complex is consistent with key features of the published mechanisms, including abstraction of H• from C5 of **3** by the ferryl intermediate and transfer of H• to the opposite side of the C5 radical by a tyrosine, but it implies that Y165, a residue not visualized in any of the published structures and therefore never invoked in the mechanisms, is the dedicated H• donor (Fig. 2C). To test these clear

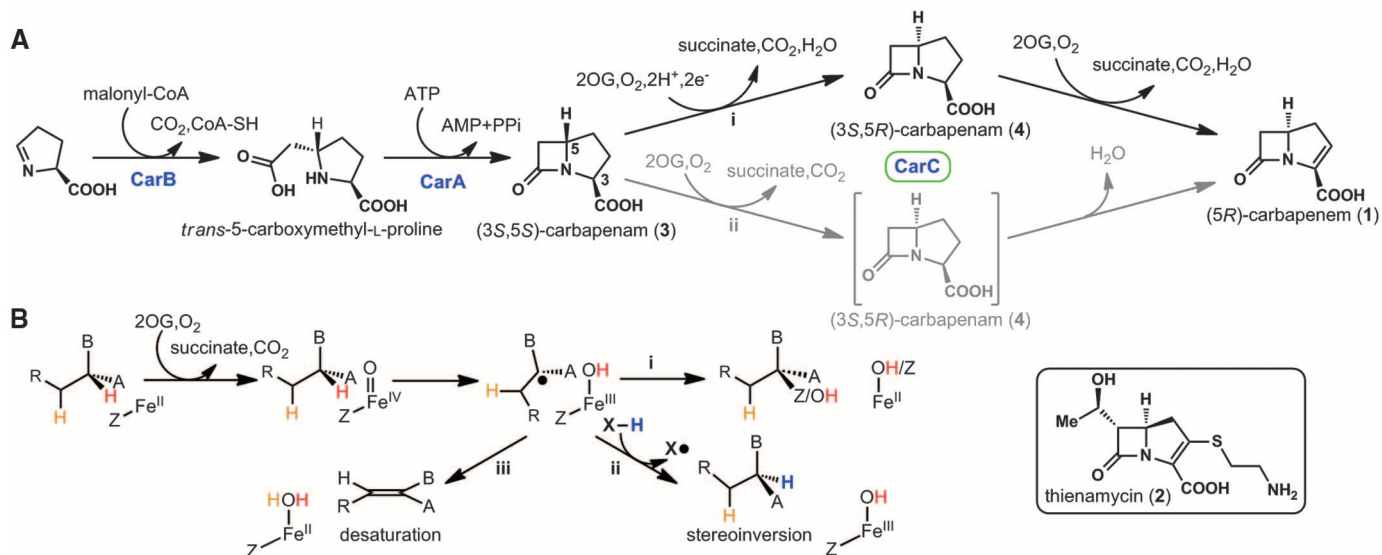


Fig. 1. Carbapenam biosynthesis, the role of CarC, and the relationship of the CarC reactions to those of other Fe/2OG oxygenases. (A) The proposed carbapenam biosynthetic pathway. **(B)** Divergence of the

(i) radical-group transfer, (ii) stereoinversion, and (iii) desaturation outcomes known or thought to be mediated by Fe/2OG oxygenases, including CarC.

functional implications, we dissected the mechanism by quantification of the reaction products (at completion and with time) and by rapid-kinetic and spectroscopic experiments on both wild-type and Y→F (phenylalanine) variant CarC proteins with both **3** and 5-*d*-**3** substrates. All results are consistent with the chemical mechanism in Fig. 2C and the kinetic elaboration of this mechanism in fig. S7. Crucial features revealed by the analysis are that the stereoinversion reaction is, in the absence of a reductant, stoichiometric (Fig. 1A, pathway i) rather than catalytic (pathway ii) as a result of oxidation of the Fe(II) cofactor to a stable Fe(III) form, and that only one-third of the enzyme's Fe(II) sites react, either because its quaternary structure (the trimeric ASU and overall hexamer seen in the crystal structure) enforces a "one-third-of-sites reactivity" or because the previously noted instability of the protein resulted in an inactive fraction. These features are most obvious from the production of a maximum of 0.32 equivalent of the succinate co-product in the presence of excess **3**, 2OG, and O₂ (fig. S8, A and B). The reaction of only one-third of the Fe(II) sites is also reflected in the accumulation

of substoichiometric quantities of the intermediate states **b** and **c** (fig. S7).

The intermediacy of a high-spin ferryl complex (Fig. 2C, state **b**), which may be general to all Fe/2OG oxygenases (22), is implied by Mössbauer spectra of a sample prepared by mixing the CarC•Fe(II)•2OG•**3** complex at 5°C with excess O₂ and freeze-quenching after 0.15 s (Fig. 3B and fig. S9). The spectrum at 4.2 K in an applied magnetic field of 53 mT (hash marks) exhibits a new feature at ~1 mm/s that is not present in the spectrum of the reactant complex (Fig. 3A). This feature is the high-energy line of a quadrupole doublet with isomer shift (δ) of 0.28 mm/s and quadrupole splitting (ΔE_Q) of 0.87 mm/s (Fig. 3B, solid line, and fig. S9), parameters very similar to those of high-spin ($S = 2$) ferryl complexes detected in other Fe/2OG oxygenases (37–41). Spectra collected in an 8-T applied field (fig. S10) confirm that the Fe(IV) complex has a high-spin ground state. The features of the complex account for 24% of the total intensity, equating to 0.24 equivalent relative to CarC•Fe(II). As noted, the modest accumulation reflects the reaction of

only one-third of the Fe(II) sites. The ferryl complex is, as expected, transient, decaying to ≤ 0.03 equivalent by a reaction time of 3 s (Fig. 3C and fig. S7). Its decay is accompanied by formation of high-spin ($S = 5/2$) Fe(III) species (Fig. 3C, arrows, and fig. S10), consistent with formation of states **c** and **d** in Fig. 2C. Conversion of the ferryl complex to accumulating Fe(III) species, which is also reflected in absorption and electron paramagnetic resonance (EPR) spectra discussed below, contrasts with events in the catalytic oxidation reactions of other well-studied Fe/2OG oxygenases, in which the ferryl complexes decay back to Fe(II) species (37, 39, 41, 42). As noted, this difference reflects the nonoxidative and stoichiometric nature of the stereoinversion reaction.

Stopped-flow absorption experiments initiated by mixing CarC•Fe(II)•2OG•**3** with excess O₂ confirm that decay of the ferryl complex between 0.15 and 3 s is associated with accumulation of a tyrosyl radical (Fig. 2C, state **c**). A sharp absorption feature at 410 nm and broader peak at 390 nm, unmistakable hallmarks of a tyrosyl radical (Y•), reach their maximum intensity at a

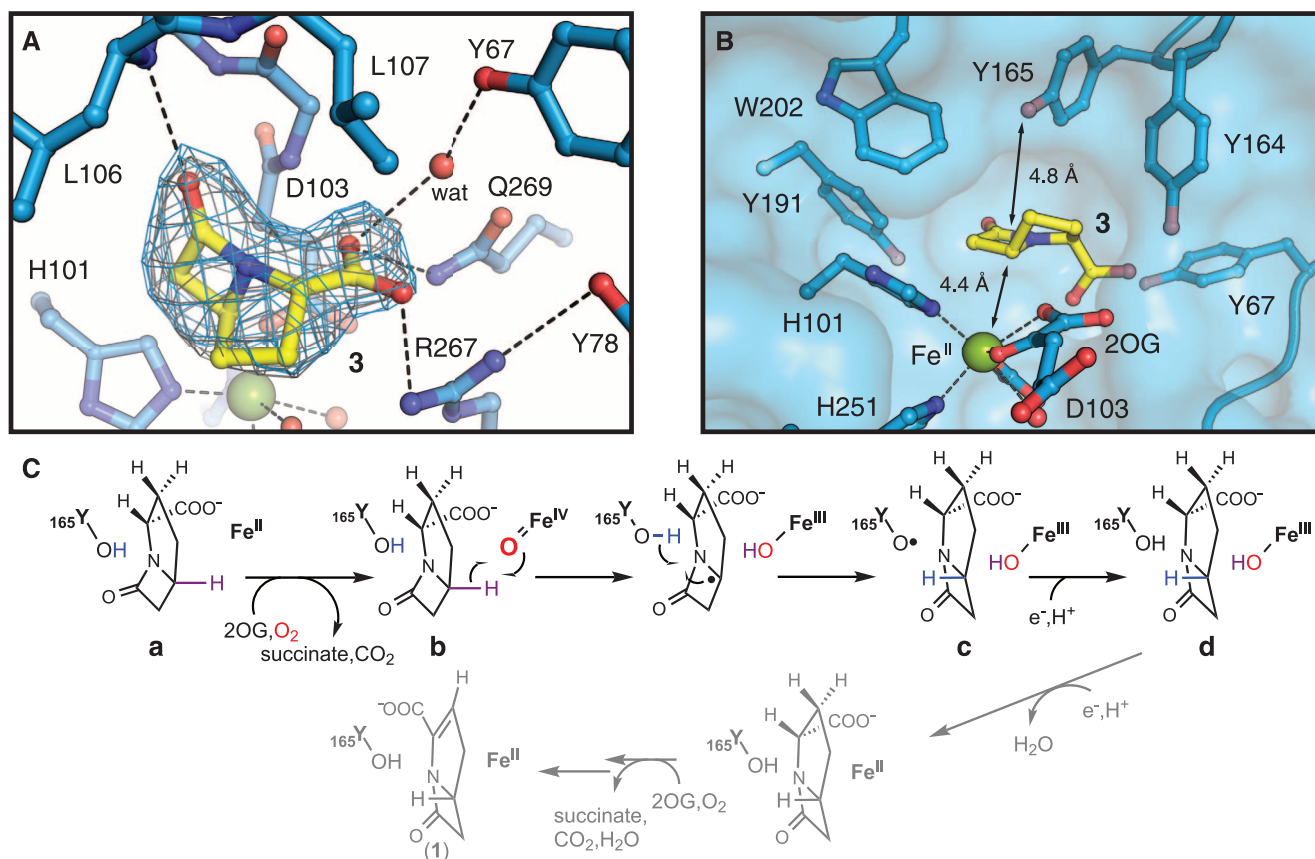


Fig. 2. Structure of the active site in the CarC•Fe(II)•2OG•3** complex and the mechanism of stereoinversion indicated by the structure and biophysical results presented in this study.** (A) $2F_o - F_c$ (gray mesh, 1.2σ) and $F_o - F_c$ omit (blue mesh, 2.6σ) electron density maps for **3** (carbon atoms shown in yellow). Dashed lines illustrate hydrogen-bonding interactions involving the substrate or bonds between the Fe(II) cofactor and its ligands. (B) Surface representation of the substrate-binding pocket showing the locations of nearby aromatic amino acids including Y165, the H• donor. (C) Mechanism of the CarC-mediated, stoichiometric stereoinversion of **3** to **4**, involving abstraction of H• from C5 by a ferryl complex and H• donation by Y165. Abbreviations for the amino acid residues are as follows: D, Asp; H, His; L, Leu; Q, Gln; R, Arg; W, Trp; and Y, Tyr.

Black lines indicate the distances between C5 of **3** and the Fe(II) or the hydroxyl substituent on Y165. Selected amino acid side chains, 2OG, and substrate **3** are shown in stick format. The Fe(II) ion and water molecules are shown as green and red spheres, respectively. (C) Mechanism of the CarC-mediated, stoichiometric stereoinversion of **3** to **4**, involving abstraction of H• from C5 by a ferryl complex and H• donation by Y165. Abbreviations for the amino acid residues are as follows: D, Asp; H, His; L, Leu; Q, Gln; R, Arg; W, Trp; and Y, Tyr.

reaction time of ~ 3 s and then decay slowly (Fig. 4, A and B). The kinetics of formation and decay of the $Y^\bullet$ (Fig. 4B, red trace), deduced by the height of the 410-nm peak (Fig. 4A, orange markings), are simulated well according to the kinetic mech-

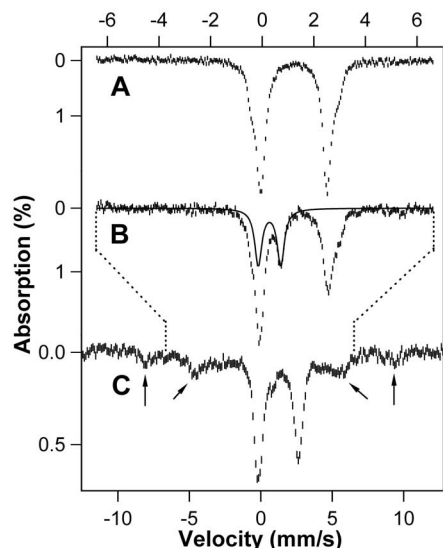


Fig. 3. Mössbauer spectra at 4.2 K and 53 mT demonstrating accumulation of an Fe(IV) intermediate (Fig. 2C, state b) and Fe(III) species (states c and d) in the CarC reaction. (A) Spectrum of the O_2 -free $CarC \cdot Fe(II) \cdot 2OG \cdot 3$ reactant solution. (B and C) Spectra of samples freeze-quenched 0.15 and 3 s, respectively, after initiating the reaction by mixing with O_2 . The solid trace is the simulated spectrum of the high-spin Fe(IV) (presumptively ferryl) species, and the arrows mark the features of the high-spin Fe(III) species. Note the different x scales for the spectra: the top scale applies to (A) and (B) and the bottom scale to (C). The dotted lines indicate the relationship of the two scales. Reaction conditions are provided in the supplementary methods.

anism in fig. S7 (compare red data and black simulation traces). The absorption in the ultraviolet region of the spectrum remaining after decay of the $Y^\bullet$ (Fig. 4A, cyan trace) reflects the conversion of the Fe(II) cofactor to Fe(III) in the stoichiometric stereoinversion, as also revealed by the Mössbauer spectra. The enzyme reacts with O_2 much less rapidly, and no $Y^\bullet$ accumulates, in analogous reactions lacking **3** in the protein reactant solution (Fig. 4B, compare red and orange traces). This activating effect of the substrate, which we have termed “substrate triggering,” is common to the Fe/2OG oxygenases (22, 39).

EPR spectra at 10 K of samples freeze-quenched at reaction times of 0.5, 3.0, and 30 s (Fig. 4C) exhibit a signal at $g = 2.0$ suggestive of an organic radical. The signal develops and decays with the same kinetics (fig. S7, red circles) as the absorption features from the $Y^\bullet$ (black trace). In the 3.0-s spectrum, the intensity of the signal reflects the accumulation of 0.25 equivalent of a radical, similar to the maximum quantity of the ferryl complex. The g -value and kinetic behavior associate it with the $Y^\bullet$, but the signal is atypical of a magnetically isolated $Y^\bullet$. It has broad features well outside the envelope of a typical $Y^\bullet$ spectrum and does not obviously exhibit the characteristic hyperfine couplings with the hydrogens on the β -carbon and phenol ring. The unusual lineshape results from magnetic interaction of the $Y^\bullet$ with the nearby high-spin Fe(III) species in state c, as explained below.

These 10-K EPR spectra have additional resonances at $g = 4.28$ and 6.95 (fig. S11). The $g = 4.28$ signal develops slowly and is then stable, and we attribute it to the stable Fe(III) cofactor form(s) in state d of the productive reaction (Fig. 2C and fig. S7) and perhaps also generated by slow oxidation of the Fe(II) in the two-thirds of the enzyme that fails to react productively. The $g = 6.95$ signal is transient and develops and decays along with the $Y^\bullet$ (fig. S11A). It arises

from the ground Kramers doublet of a high-spin ($S = 5/2$) Fe(III) center with a positive axial zero-field-splitting parameter ($D > 0$) and a rhombicity (E/D) of ~ 0.05 . Its characteristics associate it with the Fe(III) site present along with the $Y^\bullet$ in state c (Fig. 2C). Evidence for the expected interaction between the two paramagnetic species is provided by the temperature dependencies of the $g = 6.95$ and $g = 2.0$ signals. With increasing temperature from 10 to 100 K, the $g = 6.95$ signal of the Fe(III) ion becomes markedly less intense (fig. S11B). It broadens almost into the baseline at the higher temperatures as a result of more rapid spin-relaxation. Because the two species interact magnetically, the faster relaxation of the Fe(III) center affects the $g = 2.0$ signal of the $Y^\bullet$. At 100 K, the $Y^\bullet$ signal is sharper than at 10 K and clearly exhibits the characteristic hyperfine interactions with the β - and ring hydrogens (Fig. 4C, gray trace, and fig. S12). The faster spin-relaxation of the Fe(III) species at the higher temperature effectively decouples the spins of the two paramagnetic centers on the EPR time scale. The failure of the magnetic interaction with the $Y^\bullet$ to perturb the Mössbauer spectra of the Fe(III) center is explained in the supplementary materials (fig. S13).

The results of stopped-flow experiments using 5-*d*-**3** in place of **3** verify that conversion of b to c involves cleavage of the C5-H bond. The features of the $Y^\bullet$ develop more slowly and to a much lesser extent with the deuterated substrate (Fig. 4B and fig. S7, compare blue and red traces). That the $Y^\bullet$ forms more slowly reflects the deuterium kinetic isotope effect on abstraction of the C5 hydrogen and implies that $Y^\bullet$ formation follows cleavage of this bond. That much less $Y^\bullet$ accumulates implies that the H-abstracting ferryl complex can decay by at least one additional, unproductive pathway (the downward branch from b to ? in fig. S7), which contributes to a minor extent in the reaction with **3** but becomes predominant when the

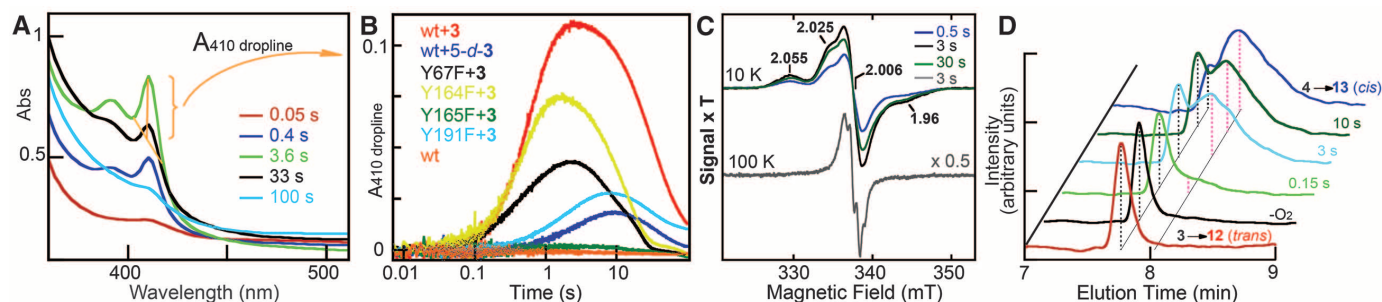


Fig. 4. Kinetic and spectroscopic evidence for state c (Fig. 2C, containing Y165 $\bullet$, Fe(III), and **4, in the CarC reaction.** (A) Change in absorbance at the indicated reaction times after mixing the $CarC \cdot Fe(II) \cdot 2OG \cdot 3$ complex with O_2 . The spectra shown were obtained by subtracting the spectrum at 5 ms from the spectra at the indicated reaction times. (B) Kinetics of $Y^\bullet$ formation and decay, as reported by the height of the sharp peak at 410 nm, in the reactions of wild-type CarC (red), and the Y67F (black), Y164F (yellow), Y165F (green), and Y191F (cyan) variants under the same conditions as in (A). The orange trace is a control reaction of the wild-type enzyme from which **3** was omitted from the protein syringe. The dark blue trace is from the reaction of the wild-type enzyme in the presence of the 5-*d*-**3** substrate. Each trace in (B) is the average of three trials constructed as indicated by the orange lines, bracket, and

arrow in (A) as $A_{410} - (A_{404} + A_{416})/2$ (A_{410} dropline). (C) X-band EPR spectra of the $Y^\bullet$ in reaction samples freeze-quenched at the indicated times. The 100-K spectrum of the 3-s sample (gray trace) has been scaled by 0.5 for clarity. Measurement conditions: microwave frequency, 9.4 GHz; microwave power, 2 μ W for 10-K spectra and 20 μ W for 100-K spectrum; modulation amplitude, 1 mT for 10-K spectra and 0.2 mT for 100-K spectrum; modulation frequency, 100 kHz. (D) Chemical-quench, LC-MS analysis of the stereoinversion of **3** to **4**. Negative-mode, single-ion chromatograms at $m/z = 172$ for acid hydrolysis of the **3** synthetic standard (**3** $\rightarrow$ **12**, red trace) and the 3.5:1 **4**:**3** synthetic standard (**4** $\rightarrow$ **13**, blue trace) and for CarC reactions acid-quenched at the indicated times. Reaction conditions for the four experiments, the procedures used to synthesize the standards, and details of the LC-MS analysis are provided in the supplementary methods.

intermediate is challenged by the heavier hydrogen isotope in 5-*d*-3. The “uncoupling” of O₂ activation from H• abstraction upon substrate deuteration has been seen in other Fe/2OG oxygenases (30, 42, 43). Accounting for the unproductive pathway(s) permits the kinetics of formation and decay of the Y• in the reaction with 5-*d*-3 (fig. S7, blue trace) to be simulated accurately (gray trace).

The results of chemical-quench kinetic experiments confirm that C5 stereoinversion also occurs in the conversion of **b** to **c**. Acid hydrolysis of **3** yields *trans*-5-carboxymethyl-L-proline, **12**, whereas hydrolysis of **4** yields the *cis* diastereomer, **13** (fig. S14A). Liquid chromatography–mass spectrometry (LC-MS) analysis of these hydrolysis products (fig. S14B, red and blue traces), along with samples of 5-carboxymethyl-L-proline enriched by synthetic methods in either **12** (purple trace) or **13** (green trace), reveals that the two ring-opened compounds can be resolved sufficiently well by hydrophilic interaction chromatography (see supplementary methods) to enable the conversion of **3** to **4** in the CarC reaction to be monitored. LC-MS analysis of a sample of the CarC•Fe(II)•2OG•**3** complex [prepared with nominally 5 molar equivalents of CarC•Fe(II) relative to **3**] denatured in formic acid without prior exposure to O₂ reveals a single sharp peak at mass/charge ratio (*m/z*) = 172.0 (Fig. 4D, black trace) coeluting at 7.8 min with the acid-hydrolyzed substrate (red trace). As expected, no peak (or shoulder) is seen at 8.0 min, when the *cis* product from hydrolysis of **4** elutes (dark blue trace). The chromatogram for a sample quenched in formic acid 3 s after the CarC•Fe(II)•2OG•**3** complex was mixed with excess O₂ (cyan trace) reveals a diminution in the peak at 7.8-min elution time corresponding to **12** (*trans*) and development of the broad peak at 8.0 min corresponding to **13** (*cis*), signifying conversion of **3** to **4**. The corresponding chromatogram from a sample acid-quenched after reacting for just 0.15 s (when state **b** predominates) has only a small shoulder for **13** (light green trace), and the trace from the 10-s sample reveals little additional conversion occurring after 3 s, the time at which state **c** accumulates maximally. The results confirm that state **b** has **3** bound to the enzyme whereas state **c** has **4** bound.

The clear implication of the CarC•Fe(II)•2OG•**3** structure—that Y165 is the H• donor and primary site of the Y•—is confirmed by the results of stopped-flow experiments on four variants of CarC each having one of the four Y residues in the halo of aromatic residues that encircles the substrate (Fig. 2B) replaced by phenylalanine, which is incompetent for H• donation. The Y67F, Y164F, and Y191F variants all form the sharp 410-nm peak of the Y• upon reaction of their enzyme•Fe(II)•2OG•**3** complexes with O₂ (Fig. 4B, black, yellow, and cyan traces, respectively). The amplitudes and kinetics are perturbed to varying extents by the substitutions, consistent with auxiliary roles for these Y residues (e.g., in binding the substrate, in ensuring the proper conformation of the H•-donating Y, or in stabilizing the Y•). By

contrast, no 410-nm signal develops in the reaction of the Y165F variant of CarC (dark green trace), and no conversion of **3** to **4** is detected in LC-MS analysis of the acid-quenched reaction products (as in Fig. 4D). These observations confirm that Y165 is the H• donor and primary site of the radical.

Figure 1B implies that H• donation to C5 diverts the CarC reaction from either of two possible catalytic oxidation outcomes (reactions **i** and **iii**) to the stoichiometric, redox-neutral stereoinversion (reaction **ii**). Consistent with this idea, the Y165F substitution defaults CarC back to a fully catalytic oxygenase. By contrast to the mediation of only 0.25 to 0.35 turnover by the wild-type enzyme, the Y165F variant protein effects minimally three turnovers under the same conditions, as seen by quantification of both succinate produced (figs. S8A, five rear traces, and S8B, red data points) and **3** consumed (fig. S8C, five rear traces). If only one-third of the Fe(II) sites react also in the Y165F variant, as suggested by the modest amplitude of decay of the ~510-nm Fe(II)→2OG metal-to-ligand charge-transfer absorption band (23) upon mixing of the CarC-Y165F•Fe(II)•2OG•**3** complex with O₂ (fig. S15), then the reactive fraction performs ~10 turnovers. The omission of the prior acid treatment in the LC-MS analysis of fig. S8 leaves **3** and **4** intact, and they are not resolved chromatographically. Under this analysis, the intensity of the single peak at *m/z* = 154 and 1.8-min elution time from the combination of **3** and **4** is not affected by the stereoinversion outcome (fig. S8C, compare red and blue traces). The almost complete diminution of this peak in samples of the CarC-Y165F reaction (fig. S8C, rear traces) thus signifies consumption of **3** by a reaction other than stereoinversion. In fig. S8D, we posit that the altered outcome is hydroxylation of C5 according to the classical “oxygen-rebound” mechanism (44), but the actual outcome remains to be determined. Regardless, the results establish that the H•-donating Y165 residue, brought into position by the substrate-driven closure of a lid loop, is necessary and sufficient to direct the nonredox, stereoinversion outcome that distinguishes CarC from other Fe/2OG oxygenases and is required for the biosynthesis of all carbapenem antibiotics.

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Supplementary Materials

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Mapping the Epigenetic Basis of Complex Traits

Sandra Cortijo,^{1*†} René Wardenaar,^{2*} Maria Colomé-Tatché,^{2*‡} Arthur Gilly,^{3§} Mathilde Etcheverry,¹ Karine Labadie,³ Erwann Caillieux,¹ Frédéric Hospital,⁴ Jean-Marc Aury,³ Patrick Wincker,^{3,5,6} François Roudier,¹ Ritser C. Jansen,² Vincent Colot,^{1||} Frank Johannes^{2||}

Quantifying the impact of heritable epigenetic variation on complex traits is an emerging challenge in population genetics. Here, we analyze a population of isogenic *Arabidopsis* lines that segregate experimentally induced DNA methylation changes at hundreds of regions across the genome. We demonstrate that several of these differentially methylated regions (DMRs) act as bona fide epigenetic quantitative trait loci (QTL^{epi}), accounting for 60 to 90% of the heritability for two complex traits, flowering time and primary root length. These QTL^{epi} are reproducible and can be subjected to artificial selection. Many of the experimentally induced DMRs are also variable in natural populations of this species and may thus provide an epigenetic basis for Darwinian evolution independently of DNA sequence changes.

Methylation of cytosines is an epigenetic mark involved in the silencing of transposable elements (TEs) and genes (1). Despite its functional conservation across many species (2, 3), intraspecific surveys have revealed widespread variation in DNA methylation patterns within populations (4–6). Estimates in the model plant *Arabidopsis thaliana* indicate that heritable changes in the methylation status of clusters of cytosines, which could be function-

ally more relevant than individual cytosines (7), arise spontaneously at rates similar to that of DNA sequence mutations (8, 9). A key challenge in population genetics is to show that epigenetic variants exist independently of cis- or trans-acting DNA sequence changes, are stably transmitted over many sexual generations, and are associated with heritable phenotypic variation (10). Addressing this challenge using natural populations continues to pose major technical difficulties.

To overcome these difficulties, we established in *Arabidopsis* a population of so-called epigenetic recombinant inbred lines (epiRILs) that have almost identical DNA sequences but segregate many differences in DNA methylation (11). To derive this population, a plant homozygous for the recessive *ddm1-2* mutation was first crossed with a near-isogenic wild-type (WT) individual. The *ddm1-2* mutation leads to a loss of DNA methylation and silencing over transposable elements (TEs) mainly, with potential consequences

¹Institut de Biologie de l'École Normale Supérieure, Centre National de la Recherche Scientifique (CNRS), UMR 8197, Institut National de la Santé et de la Recherche Médicale (INSERM) U 1024, Paris F-75005, France. ²Groningen Bioinformatics Centre, GBB, University of Groningen, 9747 AG Groningen, Netherlands. ³Commissariat à l'Energie Atomique (CEA), Institut de Génétique (IG), Genoscope, 2 Rue Gaston Crémieux, 91057 Evry, France. ⁴Institut National de la Recherche Agronomique (INRA), UMR 1313, Génétique Animale et Biologie Intégrative, Domaine de Vilvert, 78352 Jouy en Josas, France. ⁵CNRS, UMR 8030, CP5706, Evry, France. ⁶Université d'Evry, CP5706, Evry, France.

*These authors contributed equally to this work.

†Present address: Sainsbury Laboratory, University of Cambridge, Cambridge, CB2 1LR, UK.

‡Present address: European Research Institute for the Biology of Ageing, University of Groningen, UMCG, A. Deusinglaan 1, NL-9713 AV Groningen, Netherlands.

§Present address: Wellcome Trust Genome Campus, Hinxton, Cambridgeshire CB10 1SA, UK.

||Corresponding author. E-mail: colot@biologie.ens.fr (V.C.); f.johannes@rug.nl (F.J.)

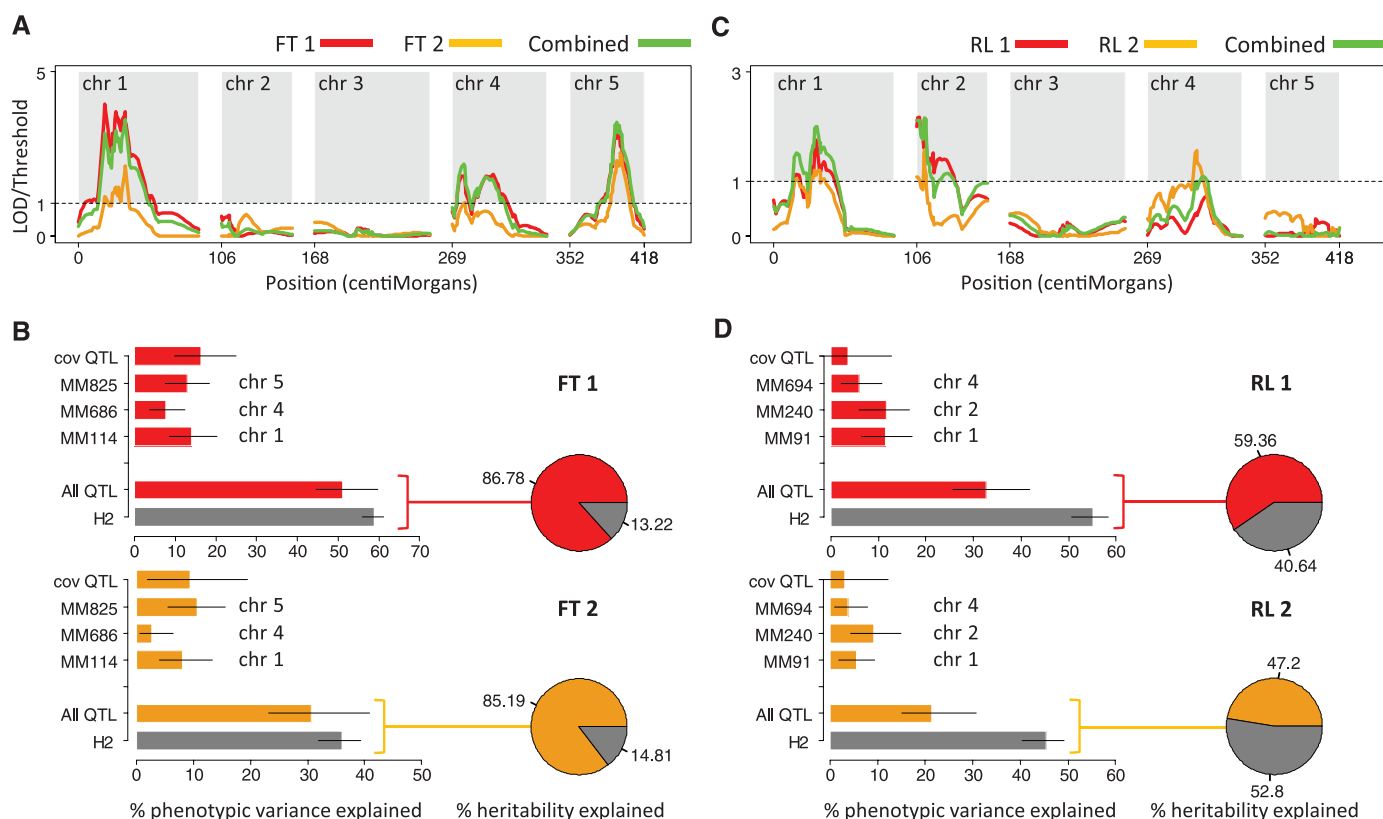


Fig. 1. Interval mapping results. (A) QTL mapping profiles for two independent flowering time measurements (FT1 and FT2), as well as their average (combined). FT1 was measured in the greenhouse and FT2 in a field experiment. (B) Percentages of phenotypic variance and of broad-sense heritability (H^2) explained

by the peak QTL markers (MM#). Error bars, ± 1 standard error of the estimate. (C) QTL mapping profiles for two independent primary root length measurements (RL1 and RL2), as well as their average (combined). Both RL1 and RL2 were measured in a climate-controlled growth chamber. (D) Same as in (B), but for RL.

on the expression of neighboring genes (12), but transposition events are relatively rare (13). Importantly, some of the DNA methylation and expression changes induced by *ddm1-2* are inherited independently of the mutation (14, 15). A single F1 *DDM1/ddm1-2* individual was therefore backcrossed to the WT parental line, and after selection of F2 progeny homozygous for the *DDM1* allele, the epiRILs ($N > 500$) were selfed for six generations (fig. S1).

Phenotypic analysis revealed significant broad-sense heritability in the epiRILs, with estimates ranging from about 0.05 to 0.4 (11, 16, 17). Theoretical predictions indicate that these heritability values are consistent with a small number of parentally derived quantitative trait loci (QTL) (17, 18). We hypothesized that these QTL are caused by stably inherited DNA methylation changes originating from the *ddm1-2* mutant founder parent. Indeed, a survey of the DNA methylomes of a selected set of 123 epiRILs identified hundreds of parental differentially methylated regions (DMRs) showing Mendelian segregation patterns (19).

Using an informative subset of 126 of these DMRs as physical markers, we were able to derive a genetic map covering 81.9% of the total genome (19).

Here, we used this map in conjunction with classical linkage analysis to search for epigenetic quantitative trait loci (QTL^{epi}) underlying complex traits in the epiRIL population.

We interval-mapped (20) two highly heritable (and weakly correlated) complex traits, flowering time (FT1) and primary root length (RL1) (Fig. 1, fig. S2, and table S1). The FT1 phenotype was obtained in a greenhouse experiment (11), whereas RL1 was measured in a climate-controlled growth chamber (21). Linkage analysis detected highly significant QTL for FT1 on chromosome 1 (chr 1) [40.59 cM; logarithm of the odds ratio for linkage (LOD) = 8.72], chr 4 (30 cM; LOD = 4.43), and chr 5 (41.73 cM; LOD = 8.53) (Fig. 1A and table S2). For these three QTL, the plants that inherited the WT epigenotype at the peak marker flowered significantly later than those with the *ddm1-2* inherited epigenotype (fig. S3A). The combined additive effects of these QTL explained 86.78%

of the broad-sense heritability for the trait and 51.14% of the total phenotypic variance (Fig. 1B and table S3). All three QTL were confirmed using independent flowering time data (FT2) collected in a field experiment (17) (Fig. 1, A and B, and table S3), which indicates that these QTL are robust across environmental settings. For RL1, we detected significant QTL on chr 1 (38 cM; LOD = 4.9), chr 2 (6.47 cM; LOD = 5.28), and chr 4 (50 cM; LOD = 2.65) (Fig. 1C and table S2). The WT-inherited epigenotype at the peak QTL markers was associated with longer primary roots compared with the *ddm1-2* inherited epigenotype (fig. S3B). The combined additive effect of these QTL explained 59.36% of the estimated broad-sense heritability and 32.69% of the total phenotypic variance (Fig. 1D and table S4). Again, all three QTL were confirmed with data from a replicate phenotyping experiment (RL2) (Fig. 1, C and D, and table S4).

Our linkage mapping results indicate that the broad-sense heritability in the epiRILs is mainly due to causal variants originating from the pa-

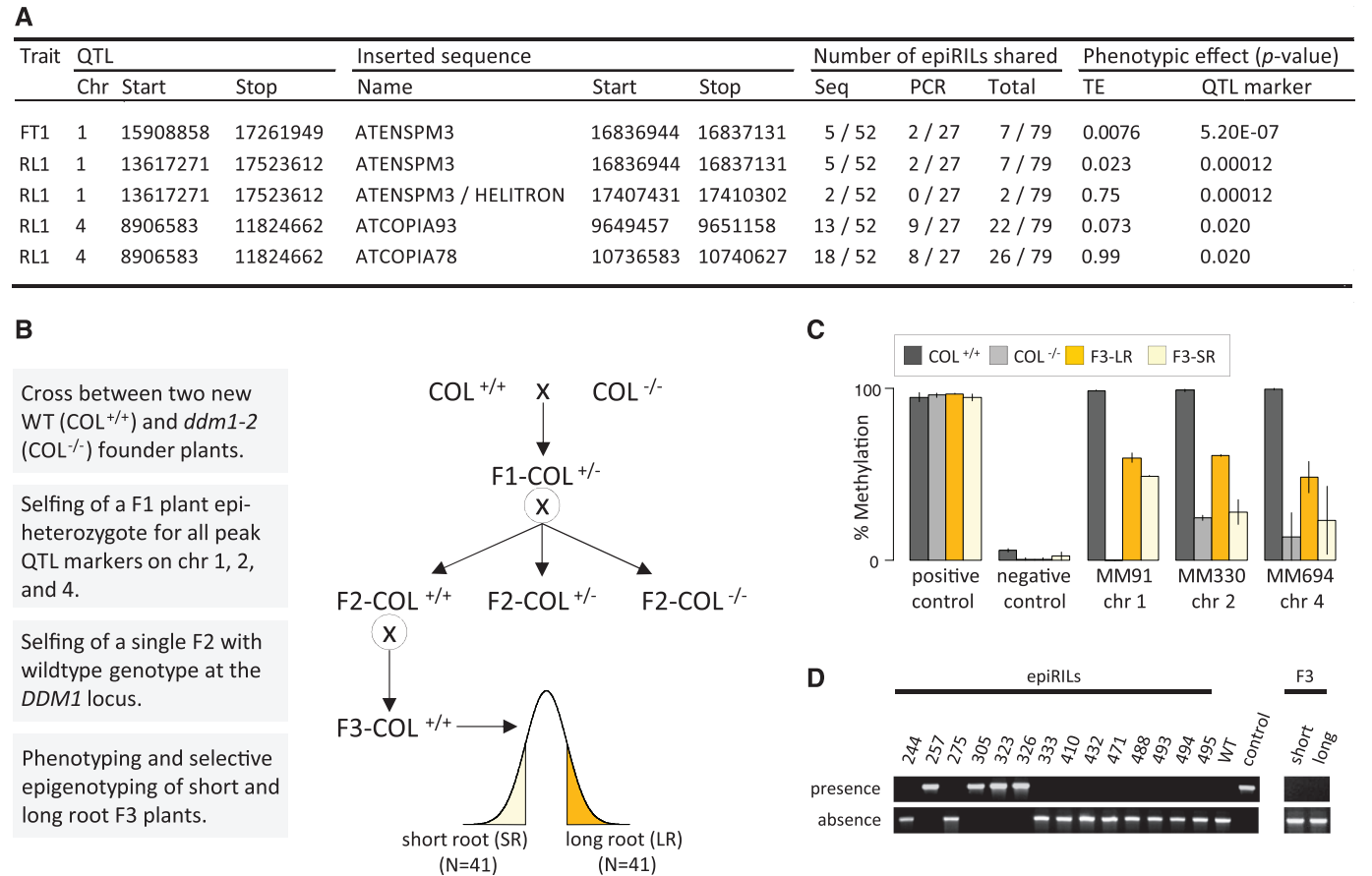


Fig. 2. Ruling out *ddm1-2*-derived TE insertions as a cause for the epiRIL QTL. (A) Resequencing of 52 epiRILs and targeted PCR of an additional 27 epiRILs detected four shared insertions in the RL and FT QTL intervals (coordinates are according to TAIR10). Phenotypic analysis testing for the effect of TEs and peak QTL markers [*P* values from multiple regression models (table S8)]. (B) Validation of the RL QTL by selective epigenotyping of 82 short- and long-root F3 progeny obtained using the crossing scheme shown. (C) Quantitative PCR analysis of MspI-digested DNA of tail-selected samples was used to determine

DNA methylation levels at the peak markers MM91, MM330, and MM694 on chr 1, 2, and 4, respectively (right panel). We used marker MM330 on chr 2 instead of the peak marker MM240. These markers are in tight linkage, but MM330 was easier to assay by PCR. Error bars, ± 1 SEM. (D) Example of the presence or absence of the most common insertion (ATCOPIA 93) in a sample of epiRILs and the pools of short- and long-root F3 individuals. (Top) Results of PCR with primer pairs designed to amplify one end of the element and its flanking sequence. (Bottom) Results of PCR with primer pairs designed to amplify the WT sequence. Positive control: epiRIL 55.

rental generation and not from later generations of inbreeding. To examine the possibility that these parentally derived causal variants are transposable element (TE) insertions that occurred in the *ddm1-2* parental line rather than DMRs, we resequenced a representative sample of 52 of the 123 epiRILs (tables S5 and S6). Our analysis revealed, in addition to several nonshared TE insertions, a total of four shared TE insertions in the RL and FT QTL confidence intervals (Fig. 2A, fig. S4, and table S6): two shared TE insertions in the chr 1 interval and two in the chr 4 interval. We were able to confirm the shared TE insertions using targeted polymerase chain reaction (PCR) assays in an additional 27 epiRILs (Fig. 2, A and D, and tables S5 to S7).

However, further analysis revealed that shared TE insertions were not consistently inherited from

the *ddm1-2* parent (fig. S4 and table S6). Thus, we found that the ATCOPIA78 insertion on chr 4 lies in an interval of WT origin and is present in about one-third of the epiRILs, which indicates that this insertion occurred in the F1 individual rather than in the *ddm1-2* parent. Similarly, the two ATENSPM3 insertions on chr 1 lie in intervals of *ddm1-2* origin but are absent in some epiRILs with this epigenotype, suggesting that they either occurred in the parental *ddm1-2* line, with excisions in some epiRILs, or else arose in the F1 individual. Finally, the ATCOPIA93 insertion on chr 4 lies in an interval that is of *ddm1-2* origin in some epiRILs and of WT origin in others, reflecting a highly dynamic inheritance pattern. Attempts to associate these shared TE insertions with phenotypes revealed a significant association for ATENSPM3 (chr 1) with root length and flowering

time (FT, $P = 0.0076$; RL, $P = 0.023$) (Fig. 2A and table S8A) and a borderline significant effect for the ATCOPIA93 (chr 4) insertion on primary root length ($P = 0.073$). However, phenotypic effects were much weaker than those of the peak QTL markers (Fig. 2A and table S8, B and C), which implies that these shared TE insertions are unlikely causal.

To support this conclusion, we crossed a new pair of WT and *ddm1-2* founder plants (Fig. 2B) (21) and selfed the F1 to select a single *DDMI/DDMI* F2 individual that was “epi-heterozygote” for all three QTL peak markers on chr 1, 2, and 4 (21). After an additional selfing, we selected F3 progeny from the long and short extremes of the RL distribution. DNA methylation analysis confirmed the association of short and long primary roots with the *ddm1-2*-like and WT methylation states at the

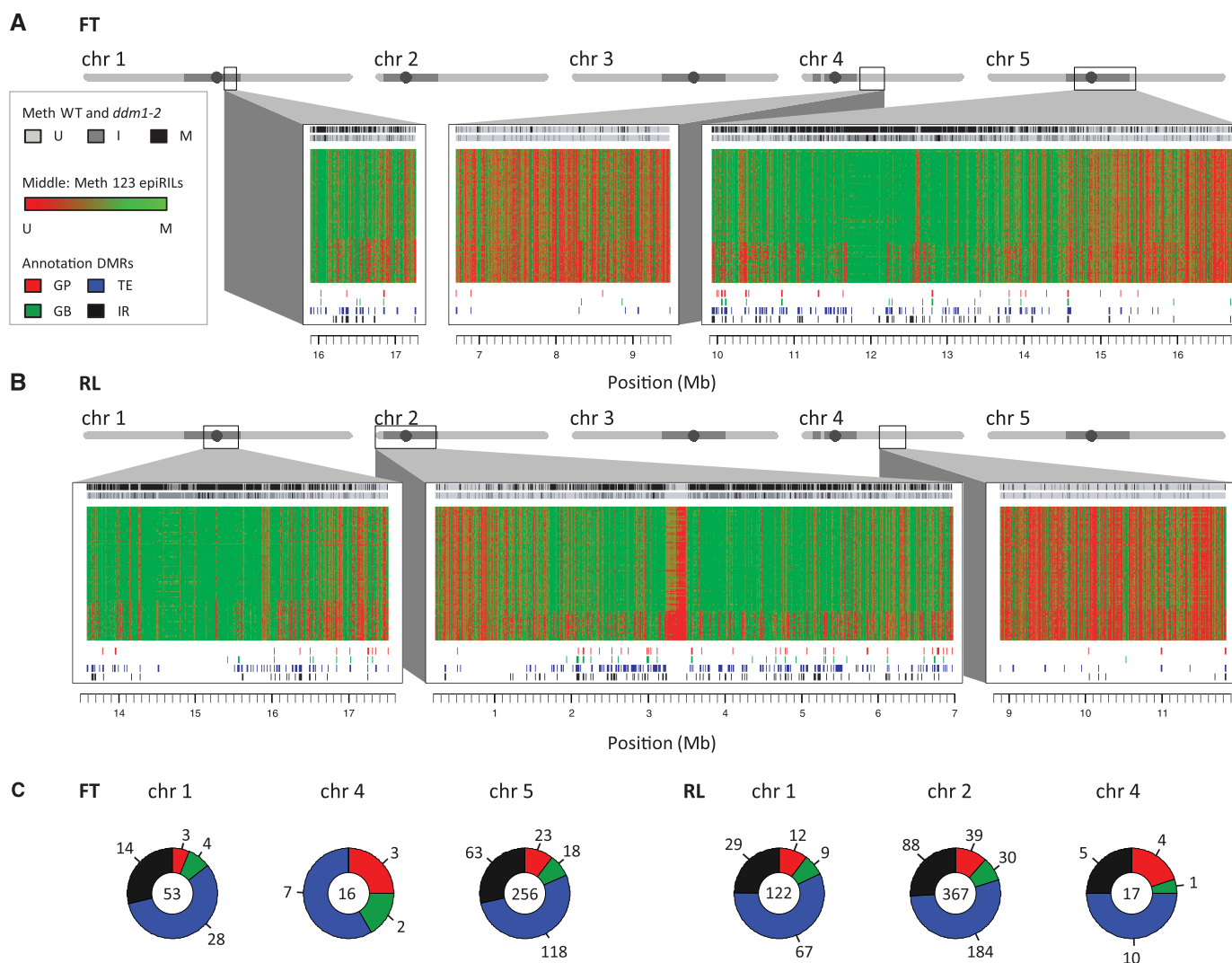


Fig. 3. DNA methylation profiles of candidate DMRs in the epiRIL QTL intervals. (A and B) Location and annotation of candidate DMRs detected for FT (A) and RL (B). The top part of the rectangles shows the DNA methylation profile of the WT and *ddm1-2* parents, respectively (U, unmethylated; I, intermediate DNA methylation; M, high-level DNA methylation). The DNA methylation profiles of the epiRILs are indicated below and are ordered according to the epigenotype of the peak marker [from WT (top) to *ddm1-2* (bottom)]. The bottom part of

the rectangles shows the annotations that overlap with the DMRs (GP, gene promoters; GB, gene bodies; TE, transposable element sequences; IR, intergenic regions). (C) Number of candidate DMRs detected for each QTL interval (values inside circles) and the number of unique annotations (values outside circles) with which they overlap. DMRs can overlap multiple annotations (tables S12 and S13). Colors and abbreviations are as in (A).

peak QTL marker, respectively (Fig. 2C) (21). Furthermore, the tail-selected F3 individuals contained none of the shared TE insertions identified in the epiRIL population (Fig. 2D and table S6). We thus conclude that the epiRIL QTL are most likely caused by the heritable (*ddm1-2* induced) loss of DNA methylation in the QTL intervals.

Next, we searched for putative causal DMRs in the RL and FT QTL intervals. We analyzed the methylomes (~165–base pair resolution) of the 123 epiRILs and their founders (19) and required candidate DMRs to be in approximate linkage disequilibrium with the peak QTL marker and displaying clear differences in DNA methylation states and expression levels between the WT and the *ddm1-2* founder lines (figs. S5 to S8). Our search revealed 325 candidate DMRs within the FT QTL intervals (chr1, 53; chr4, 16; chr5, 256), which mapped to 44 unique genes (including promoter regions), 153 annotated TE sequences, and 77 intergenic regions (Fig. 3, A and C, and tables S9, S10, and S12). For the RL QTL intervals, we detected 506 candidate DMRs (chr 1, 122; chr2, 367; chr4, 17), mapping to 71 unique genes (including promoter regions), 261 annotated TE sequences, and 122 intergenic regions (Fig. 3, B and C, and tables S9, S11, and S13). Further analysis of these candidate DMRs did not identify any obvious flowering time and root length genes (tables S10 and S11), which could be consistent with the lower amplitude of phenotypic variation observed among the epiRILs than among highly contrasted accessions (16). However, we cannot rule out that the candidate DMRs are in LD with causal DMRs that could not be called with our method. Ultimately, fine-mapping approaches and targeted manipulation

of selected DMRs will be required to identify causal regions.

Our analysis of the epiRILs demonstrates that induced DMRs can be stably inherited independently of DNA sequence changes and function as epigenetic quantitative trait loci (QTL^{epi}). Phenotypically, the detected QTL^{epi} have all the necessary properties to become targets of natural or artificial selection. Taking advantage of the single-nucleotide resolution methylomes of 138 natural accessions (4) (table S14), we could show that about 30% of the heritable DMRs identified in the epiRIL population overlap with naturally occurring DMRs among these accessions (figs. S9 and S10). Therefore, these epiRIL DMRs may have been historical targets of epimutations in the wild, either through trans-induced *ddm1*-like mutation events or else through still unknown mechanisms. This finding indicates in turn that DMRs could also act as QTL^{epi} in natural populations and thus constitute a measurable component of the so-called “missing heritability.” This possibility may have deep implications on how we delineate and interpret the heritable basis of complex traits.

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A Single Gene Affects Both Ecological Divergence and Mate Choice in *Drosophila*

Henry Chung,¹ David W. Loehlin,¹ Héloïse D. Dufour,¹ Kathy Vaccarro,¹ Jocelyn G. Millar,² Sean B. Carroll^{1*}

Evolutionary changes in traits involved in both ecological divergence and mate choice may produce reproductive isolation and speciation. However, there are few examples of such dual traits, and the genetic and molecular bases of their evolution have not been identified. We show that methyl-branched cuticular hydrocarbons (mbCHCs) are a dual trait that affects both desiccation resistance and mate choice in *Drosophila serrata*. We identify a fatty acid synthase *mFAS* (*CG3524*) responsible for mbCHC production in *Drosophila* and find that expression of *mFAS* is undetectable in oenocytes (cells that produce CHCs) of a closely related, desiccation-sensitive species, *D. birchii*, due in part to multiple changes in cis-regulatory sequences of *mFAS*. We suggest that ecologically influenced changes in the production of mbCHCs have contributed to reproductive isolation between the two species.

The evolution of traits with dual roles in ecological divergence and mate choice could cause populations to become reproductively isolated through local adaptation (1, 2).

However, the direct contribution of ecological divergence to speciation is uncertain, in part because relatively few systems have been investigated experimentally, and the genes affecting

dual traits have not been identified. Insect cuticular hydrocarbons (CHCs) are potential dual traits (3). CHCs seal the cuticle, protecting against desiccating environments (4), and can also act as pheromones that influence mate choice and mating success (5). Whereas specific CHC molecules act as pheromones (6, 7), no specific CHC or class of CHCs has been demonstrated to be involved directly in desiccation resistance.

One class of CHCs, methyl-branched CHCs (mbCHCs), which have melting points above ambient temperature, are hypothesized to help maintain a barrier against evaporative water loss (8). *D. serrata*, a habitat generalist found outside of and on the fringes of the rainforest on the east coast of Australia, is relatively desiccation resistant and produces relatively large amounts of mbCHCs (29% of all CHCs) (Fig. 1A). In contrast, its close relative *D. birchii*, a habitat specialist found exclusively in the humid rainforest, is ex-

¹Howard Hughes Medical Institute and Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706, USA. ²Department of Entomology, University of California, Riverside, CA 92521, USA.

*Corresponding author. E-mail: sbcarroll@wisc.edu



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peak QTL marker, respectively (Fig. 2C) (21). Furthermore, the tail-selected F3 individuals contained none of the shared TE insertions identified in the epiRIL population (Fig. 2D and table S6). We thus conclude that the epiRIL QTL are most likely caused by the heritable (*ddm1-2* induced) loss of DNA methylation in the QTL intervals.

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A Single Gene Affects Both Ecological Divergence and Mate Choice in *Drosophila*

Henry Chung,¹ David W. Loehlin,¹ Héloïse D. Dufour,¹ Kathy Vaccarro,¹ Jocelyn G. Millar,² Sean B. Carroll^{1*}

Evolutionary changes in traits involved in both ecological divergence and mate choice may produce reproductive isolation and speciation. However, there are few examples of such dual traits, and the genetic and molecular bases of their evolution have not been identified. We show that methyl-branched cuticular hydrocarbons (mbCHCs) are a dual trait that affects both desiccation resistance and mate choice in *Drosophila serrata*. We identify a fatty acid synthase *mFAS* (*CG3524*) responsible for mbCHC production in *Drosophila* and find that expression of *mFAS* is undetectable in oenocytes (cells that produce CHCs) of a closely related, desiccation-sensitive species, *D. birchii*, due in part to multiple changes in cis-regulatory sequences of *mFAS*. We suggest that ecologically influenced changes in the production of mbCHCs have contributed to reproductive isolation between the two species.

The evolution of traits with dual roles in ecological divergence and mate choice could cause populations to become reproductively isolated through local adaptation (1, 2).

However, the direct contribution of ecological divergence to speciation is uncertain, in part because relatively few systems have been investigated experimentally, and the genes affecting

dual traits have not been identified. Insect cuticular hydrocarbons (CHCs) are potential dual traits (3). CHCs seal the cuticle, protecting against desiccating environments (4), and can also act as pheromones that influence mate choice and mating success (5). Whereas specific CHC molecules act as pheromones (6, 7), no specific CHC or class of CHCs has been demonstrated to be involved directly in desiccation resistance.

One class of CHCs, methyl-branched CHCs (mbCHCs), which have melting points above ambient temperature, are hypothesized to help maintain a barrier against evaporative water loss (8). *D. serrata*, a habitat generalist found outside of and on the fringes of the rainforest on the east coast of Australia, is relatively desiccation resistant and produces relatively large amounts of mbCHCs (29% of all CHCs) (Fig. 1A). In contrast, its close relative *D. birchii*, a habitat specialist found exclusively in the humid rainforest, is ex-

¹Howard Hughes Medical Institute and Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53706, USA. ²Department of Entomology, University of California, Riverside, CA 92521, USA.

*Corresponding author. E-mail: sbcarroll@wisc.edu

tremely sensitive to desiccation and produces very low amounts of mbCHCs (9–11) (3% of all CHCs) (Fig. 1 and fig. S1A). *D. serrata* and *D. birchii* show prezygotic isolation, which appears to be mediated by chemical signals (12). Notably, mbCHC levels are one factor correlated with male mating success in *D. serrata* (13, 14). If mbCHCs contribute to both mating success and desiccation resistance, then mbCHCs would constitute a dual trait, and the loss of mbCHCs in *D. birchii* could have contributed to its reproductive isolation from *D. serrata*.

To explore this scenario, we identified the gene(s) responsible for the differences in mbCHCs between *D. serrata* and *D. birchii*. Because the levels of both major mbCHCs (2Me-C26 and 2Me-C28) are lower in *D. birchii* than in *D. serrata* (10) (fig. S1), we reasoned that the underlying genetic differences between these two species must have occurred early in the fatty acid synthase (FAS) pathway responsible for mbCHC synthesis (Fig. 1B).

Biochemical studies have identified two forms of FASs in insects, one for producing unbranched CHCs such as alkanes, monoenes, and dienes, and the other for producing mbCHCs (15). The *D. melanogaster* genome contains three putative FASs: *CG3523*, *CG3524*, and *CG17374* (16). In situ hybridization of RNA probes for all three FASs in adult abdomens revealed that *CG3523* is expressed only in the adult fat body, whereas *CG17374* and *CG3524* are both expressed in adult

D. melanogaster oenocytes (Fig. 1C), where CHCs are produced (17).

RNA interference (RNAi)-mediated knockdown of *CG3524* and *CG17374* in *D. melanogaster* by a ubiquitously expressed driver (tubulin-GAL4) was lethal. An oenocyte-specific driver (*oenGAL4*) (17) inducing *CG17374* RNAi was also lethal, whereas *oenGAL4*-driven RNAi knockdown of *CG3524* produced viable adult flies in which mbCHC production was strongly and specifically reduced (Fig. 1D). This suggests that *CG3524* is a mbCHC-specific FAS (*mFAS*). RNAi-mediated knockdown of *CG17374* in oenocytes after adult flies eclosed yielded viable adults with unchanged CHC profiles, whereas knockdown of *CG3524* in adults reduced mbCHCs, thus confirming the specific role of *CG3524* as *mFAS*.

We then isolated the orthologous gene in the *D. serrata* genome and drove *D. serrata* *mFAS* RNAi under the direct control of an oenocyte-specific enhancer (fig. S2), obtaining two independent lines of transgenic *D. serrata* flies carrying this construct (*mFAS^{RNAi-1}* and *mFAS^{RNAi-2}*). mbCHC levels were reduced in males and females of both lines, indicating that the role of *mFAS* in producing mbCHCs is conserved among *Drosophila* (Fig. 2A and table S1).

To assess whether mbCHCs contribute to desiccation resistance in *D. serrata*, we subjected *D. serrata* *mFAS*-RNAi and wild-type flies to a desiccation assay. We found that the *D. serrata* *mFAS*-RNAi flies exhibited reduced desiccation

resistance (Fig. 2B). To investigate whether the loss of desiccation resistance was due to the specific loss of mbCHCs, we applied a mixture of purified synthetic mbCHCs to *D. serrata* *mFAS*-RNAi flies. The application of synthetic mbCHCs significantly increased resistance (fig. S3B), indicating that the low resistance of *mFAS*-RNAi flies was due to the specific loss of mbCHCs. We also applied synthetic mbCHCs to *D. birchii* flies and observed increased desiccation resistance (fig. S3B), suggesting that the comparatively low desiccation resistance of this species may be due to at least in part to its low levels of mbCHCs.

The *D. birchii* *mFAS* gene (GenBank JX627578) contains a complete coding sequence, and cDNA could be isolated from adults, indicating that the gene is intact and functional. However, *mFAS* expression was not detectable in *D. birchii* oenocytes by in situ hybridization, suggesting that transcription in these cells is reduced or absent (Fig. 3). *mFAS* is expressed in the oenocytes of all other *Drosophila* species examined, including *D. serrata* (fig. S4). Thus, *mFAS* expression in oenocytes appears to have been lost in the *D. birchii* lineage.

To determine whether loss of *mFAS* expression is due to cis-regulatory changes, we made green fluorescent protein (GFP) reporter constructs with segments of the *mFAS* gene from both species and compared their activity when transformed into *D. melanogaster*. Both 5' and intronic *mFAS* sequences from *D. birchii* exhibited lower activity

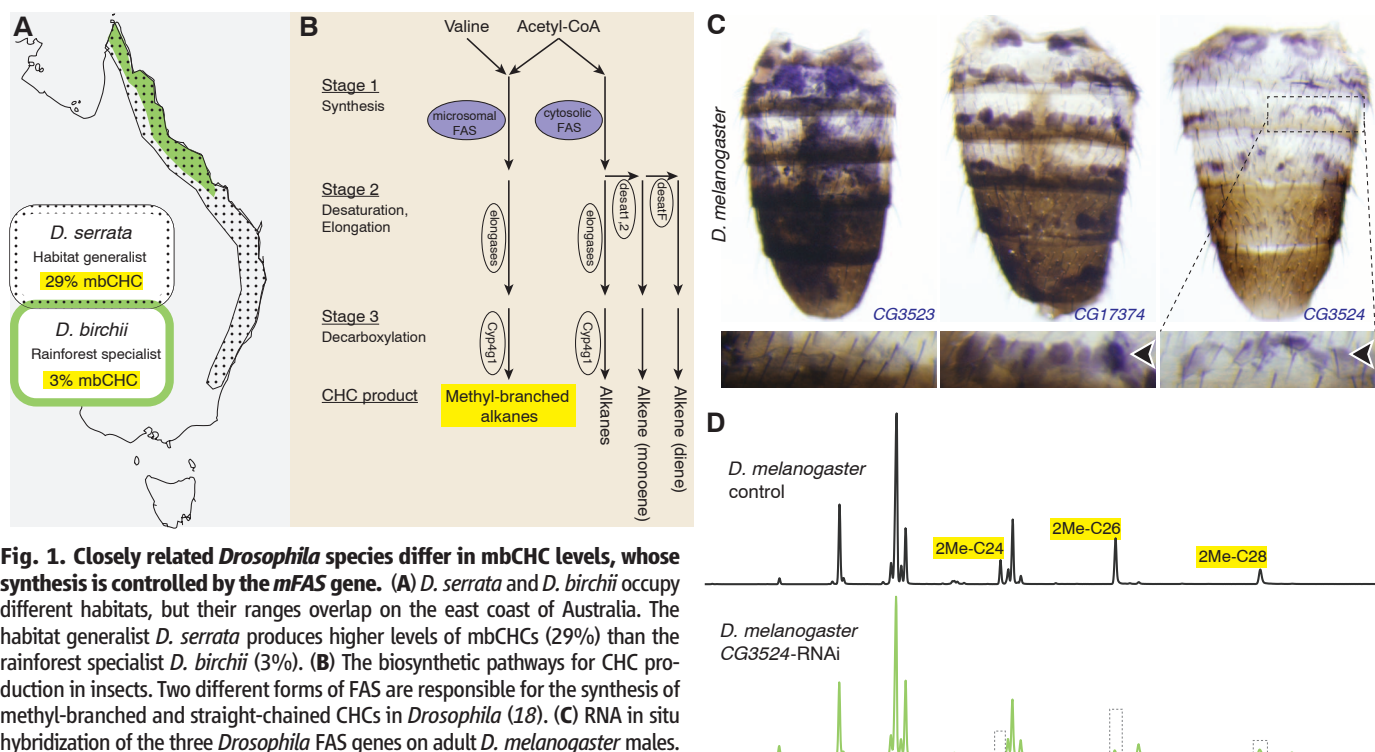


Fig. 1. Closely related *Drosophila* species differ in mbCHC levels, whose synthesis is controlled by the *mFAS* gene. (A) *D. serrata* and *D. birchii* occupy different habitats, but their ranges overlap on the east coast of Australia. The habitat generalist *D. serrata* produces higher levels of mbCHCs (29%) than the rainforest specialist *D. birchii* (3%). (B) The biosynthetic pathways for CHC production in insects. Two different forms of FAS are responsible for the synthesis of methyl-branched and straight-chained CHCs in *Drosophila* (18). (C) RNA in situ hybridization of the three *Drosophila* FAS genes on adult *D. melanogaster* males. *CG3523* transcript was detected in the fat body. *CG17374* and *CG3524* transcripts were detected in the oenocytes (arrowheads), the site of CHC synthesis. Insets show right side of second tergite (abdominal segment). For the *CG3523* inset, the fat body was removed. Expression of all three FASs is sexually monomorphic. (D)

Oenocyte-specific RNAi knockdown of *CG3524* in *D. melanogaster* results in a large reduction of mbCHCs (results shown in males), suggesting that it is a methyl-branched specific FAS (*mFAS*).

than the homologous regions from *D. serrata* (fig. S5), indicating that the reduction in *D. birchii* *mFAS* expression is partly due to multiple cis-regulatory changes at the locus.

The ecological divergence of the rainforest-restricted *D. birchii* and widely distributed *D. serrata* involved reductions of desiccation resistance, *mFAS* expression, and mbCHCs. mbCHCs are one factor that has been correlated with male mating success in *D. serrata* (13, 14), and thus could have played a role in reproductive isolation between the species. To determine whether mbCHCs directly affect mating in *D. serrata*, we conducted a series of mate-choice experiments in which mbCHC levels were manipulated. When single wild-type males were presented with a choice between a wild-type female and a *mFAS*-RNAi female, we did not observe any mating preference ($n = 105$; $\chi^2 = 0.77$; $P = 0.38$). This suggests that *D. serrata* males do not discriminate on the basis of mbCHC levels in females. In contrast, when single wild-

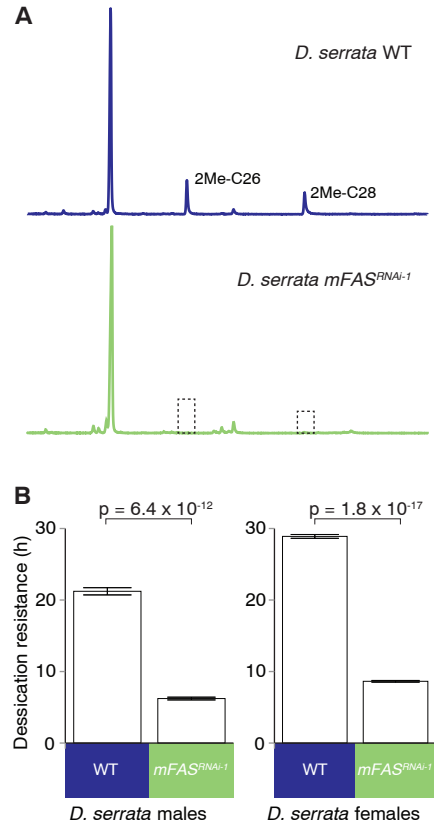


Fig. 2. Loss of mbCHCs strongly reduces desiccation resistance in *D. serrata*. (A) Oenocyte-specific RNAi knockdown of *mFAS* in *D. serrata* results in almost complete loss of mbCHCs (results shown in males). (B) Oenocyte-specific RNAi knockdown of *mFAS* in *D. serrata* results in a dramatic reduction of desiccation resistance in both male (t test; $df = 17$; $t = 26.7$, $P = 6.4 \times 10^{-12}$) and female ($df = 18$; $t = 65.7$; $P = 1.8 \times 10^{-17}$) *mFAS*^{RNAi-1} flies. The mean time to mortality of 10 flies in each of $n = 9$ to 10 vials per genotype was compared with t tests (two-tailed, unpaired, unequal variance). Mean $\pm$ SEM is shown.

type females were given the choice between a wild-type male and either a *mFAS*^{RNAi-1} or *mFAS*^{RNAi-2} male, they preferred males with normal production of mbCHCs over males with reduced production (Table 1) [this effect was dependent on the age of the flies (see table S2)].

To determine whether increasing the level of mbCHCs also influenced mating success, we perfumed wild-type males with either 2Me-C26 or 2Me-C28 (table S3). We found that males perfumed with 2Me-C26 exhibited increased mating success, whereas 2Me-C28 had no detectable effect (Table 1). Finally, we investigated whether perfuming *mFAS*-RNAi males with either 2Me-C26 or 2Me-C28 increased male mating success

and found that 2Me-C26 again increased male mating success. Together, our results indicate that mbCHCs, whose production is controlled by the *mFAS* gene, affect mating success as well as desiccation resistance, and thus function as a dual trait in *D. serrata*.

To determine whether the difference in mbCHC levels might also contribute to premating isolation between *D. serrata* and *D. birchii*, we investigated whether manipulating the level of mbCHCs in *D. birchii* could break the interspecies mating barrier. We perfumed *D. birchii* males and females with synthetic mbCHCs before introducing them to *D. serrata* females and males, respectively. In neither case did we observe any copulations. Importantly, however, we also observed that no males of either species even attempted to court females of the other species, a necessary preamble before females exhibit a choice. These observations suggest that courtship by males is also governed by cues that differ between the species, such that modulation of mbCHCs alone is not sufficient to surmount the barrier to mating. This is not surprising because numerous factors contribute to courtship and mating among *Drosophila* species, and these traits may also be subject to rapid divergence (18, 19).

Although restoring mbCHCs to *D. birchii* was not sufficient to surmount its reproductive isolation from *D. serrata*, the demonstrated role of mbCHCs as a component of mate choice in *D. serrata* and the loss of mbCHCs in its rainforest-restricted sibling suggest a plausible scenario for mbCHCs in ecological speciation. That is, as populations of the common ancestor of these two species diverged ecologically, and if the rainforest-adapting population lost mbCHCs and *mFAS* expression before or during speciation (perhaps due to relaxed constraints on desiccation resistance), then such changes would likely result in increasing reproductive isolation between the populations. Subsequent changes at other genetic loci would then complete and/or reinforce speciation (fig. S6).

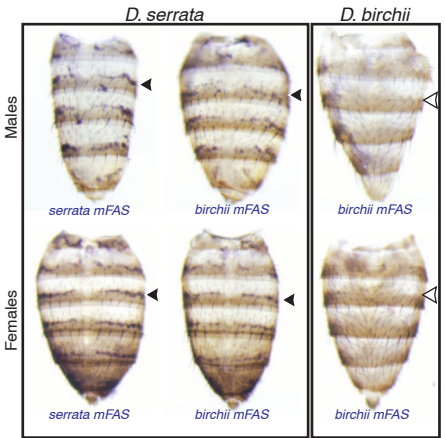


Fig. 3. *mFAS* is not expressed in *D. birchii* oenocytes. RNA in situ hybridization reveals that, whereas *mFAS* shows a sexually monomorphic expression pattern in *D. serrata* oenocytes (left), it is undetectable in either sex in *D. birchii* (right). To confirm that the lack of transcript detection was not due to any defect of the *D. birchii* RNA probe, we showed that this probe detected *mFAS* expression in *D. serrata* (center). Arrowheads point to oenocytes; open arrowheads indicate no visible expression.

Table 1. mbCHCs affect mating success in *D. serrata*. (A) Three-day-old wild-type *D. serrata* males had significantly higher mating success than *mFAS*-RNAi flies in female choice assays where single wild-type females were given a choice between the two genotypes. (B) Wild-type and *mFAS*^{RNAi-2} males were perfumed with 2Me-C26 and 2Me-C28 individually. Females are given a choice between a perfumed male and a nonperfumed male with the same genotype. Perfuming with 2Me-C26 significantly increased mating success of both wild-type and *mFAS*-RNAi flies.

(A) Wild-type (WT) males have greater mating success than <i>mFAS</i> -RNAi males						
	<i>n</i>	WT chosen	RNAi chosen	<i>df</i>	χ^2	<i>P</i>
WT versus <i>mFAS</i> ^{RNAi-1}	79	55	24	1	12.165	<0.001***
WT versus <i>mFAS</i> ^{RNAi-2}	89	54	35	1	4.056	<0.05*

(B) Males perfumed with 2Me-C26 have greater mating success than nonperfumed flies						
	<i>n</i>	Perfumed chosen	Nonperfumed chosen	<i>df</i>	χ^2	<i>P</i>
WT (2Me-C26)	141	83	58	1	4.433	<0.05*
WT (2Me-C28)	122	60	62	1	0.330	0.86
<i>mFAS</i> ^{RNAi-2} (2Me-C26)	103	71	32	1	14.767	<0.001***
<i>mFAS</i> ^{RNAi-2} (2Me-C28)	100	49	51	1	0.040	0.85

It is becoming increasingly apparent that adaptation to different environments may lead to reproductive isolation (*I*). Given the dual role of CHCs in insect water balance and sexual communication, and their great chemical diversity, changes in CHC production in ecologically diverging populations may be an important general contributor to insect speciation.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S6
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References (20–29)

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Long-Acting Integrase Inhibitor Protects Macaques from Intrarectal Simian/Human Immunodeficiency Virus

Chasity D. Andrews,¹ William R. Spreen,² Hiroshi Mohri,¹ Lee Moss,² Susan Ford,² Agegnehu Gettie,¹ Kasi Russell-Lodrigue,³ Rudolf P. Bohm,³ Cecilia Cheng-Mayer,¹ Zhi Hong,² Martin Markowitz,¹ David D. Ho^{1*}

GSK1265744 (GSK744) is an integrase strand-transfer inhibitor that has been formulated as a long-acting (LA) injectable suitable for monthly to quarterly clinical administration. GSK744 LA was administered at two time points 4 weeks apart beginning 1 week before virus administration, and macaques were challenged weekly for 8 weeks. GSK744 LA, at plasma concentrations achievable with quarterly injections in humans, protected all animals against repeated low-dose challenges. In a second experiment, macaques were given GSK744 LA 1 week before virus administration and challenged repeatedly until infection occurred. Protection decreased over time and correlated with the plasma drug levels. With a quarterly dosing schedule in humans, our results suggest that GSK744 LA could potentially decrease adherence problems associated with daily preexposure prophylaxis (PrEP).

The global HIV-1 pandemic remains a public health problem of unprecedented proportions. There were 2.3 million new infections in 2012, with an estimated 35.3 million people already infected (*1*). Because an effective vaccine remains elusive, multiple clinical trials have been performed to evaluate the efficacy of various antiretrovirals as preexposure prophylaxis (PrEP). The CAPRISA 004, the Chemoprophylaxis for HIV Prevention in Men (iPrEx), the PartnersPrEP, and the TDF2 studies have shown that topical or oral PrEP agents reduced the risk of HIV-1 infection, with efficacies ranging from 39 to 75%

(2–5). The variability in efficacy likely resulted from adherence differences among the study populations. In the iPrEx study, the efficacy of daily oral emtricitabine and tenofovir disoproxil fumarate (FTC/TDF) increased from 44% in all participants assigned to treatment to 90% in those participants with detectable drug in plasma (3). In a follow-up pharmacokinetic (PK) study, intracellular drug concentrations achieved from two, four, or seven directly observed doses of oral FTC/TDF per week correlated with 76%, 96%, or 99% efficacy in the iPrEx study, respectively (6). In contrast, the FEM-PrEP and the VOICE PrEP trials were stopped because of futility, and in both studies plasma drug concentrations revealed that <30% of participants were adherent to the dosing regimen (7, 8).

We hypothesize that long-acting (LA) antiretroviral formulations requiring infrequent dosing

could improve adherence and thus PrEP efficacy. GSK1265744 (GSK744) is an analog of dolutegravir, a recently approved integrase strand-transfer inhibitor with a favorable efficacy and safety profile in several treatment trials (9–15). About 2600 patient years of exposure (median duration of 590 days, range from 1 to 1031) were accrued during phase 2b to 3b dolutegravir clinical trial programs. GSK744 is potent in vitro with a median inhibitory concentration (IC₅₀) of 0.22 nM against HIV-1 BAL in human peripheral blood mononuclear cells (PBMCs) (16). However, GSK744 is highly protein-bound, thereby shifting up the target IC₅₀ 408-fold in the presence of human serum (17). In infected patients treated daily with 5 or 30 mg of oral GSK744 monotherapy for 10 days, mean plasma trough concentrations exceeded the protein-adjusted levels that would block 90% of infections (PAIC₉₀) by 3.5- and 20-fold, respectively, resulting in a 2.2- to 2.3-log₁₀ decrease in plasma HIV-1 RNA without the emergence of drug resistance mutations (18).

GSK744's high potency, low aqueous solubility, slow metabolism, and high melting point permitted its formulation as a 200-mg/ml LA injectable product. In healthy volunteers, GSK744 LA yielded an apparent terminal-phase half-life (*t*_{1/2}) ranging from 21 to 50 days, compared with about 40 hours for a single oral dose (16, 18). This increase resulted from a slower release of drug from the injected nanosuspension rather than a change in the metabolic elimination rate. Safety analyses across six GSK744 oral studies and two GSK744 LA studies, dosing a total of 245 participants (65 females, 180 males; median age of 32 years), revealed drug-related adverse events (dizziness and grade 1 rash) in only two participants, without any drug-related grade 3 or 4 adverse events or deaths (19). Dose-independent injection site reactions were common after GSK744 LA administration, but these were generally

¹Aaron Diamond AIDS Research Center, Rockefeller University, New York, NY 10016, USA. ²GlaxoSmithKline, Research Triangle Park, NC 27709, USA. ³Tulane National Primate Research Center, Covington, LA 70433, USA.

*Corresponding author. E-mail: dho@adarc.org

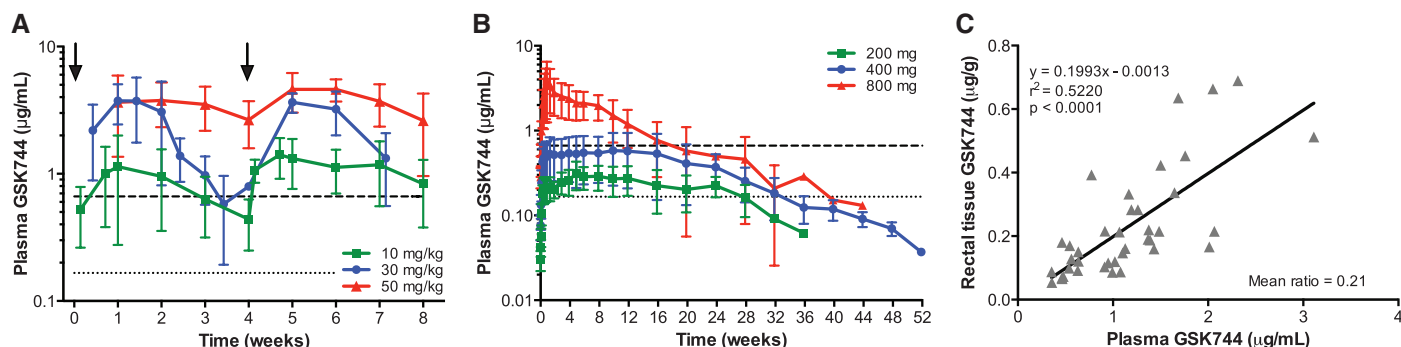


Fig. 1. GSK744 LA PK profile in rhesus macaques and humans. (A) GSK744 LA PKs were evaluated in rhesus macaques ($n = 8$) after intramuscular injections of 10 mg/kg into quadriceps as a single injection, 30 mg/kg ($n = 4$, two injections of 15 mg/kg) as a split injection, or 50 mg/kg ($n = 8$, four of 12.5 mg/kg) as a split dose with four injections, two per muscle. GSK744 LA was administered on weeks 0 and 4 (black arrows). (B) GSK744 LA single-dose PKs were evaluated in a phase 1 study, and the results were adapted here for comparison (16, 21). GSK744 LA was administered by intramuscular gluteal injection of 200 ($n = 6$), 400 ($n = 14$), or 800 ($n = 6$, two of 400 mg) mg to healthy human participants. Plasma GSK744 concentrations

were assessed by liquid chromatography–mass spectrometry (HPLC–MS/MS) with a limit of quantitation (LOQ) >0.01 $\mu\text{g/mL}$. Means $\pm$ SDs are shown. Dotted and dashed horizontal lines represent $1\times$ and $4\times$ PAIC₉₀, respectively. (C) Rectal tissue distribution of GSK744 was evaluated in macaques dosed with 10 or 30 mg/kg of GSK744 LA. The drug concentration from pinch mucosal biopsies was assessed each week in a subset ($n = 4$) of animals injected with 10 mg/kg. GSK744 concentration was assessed in all animals ($n = 4$) treated with 30 mg/kg of GSK744 LA at weeks 2, 4, and 7. Tissue concentrations were assessed by HPLC–MS/MS with LOQ >0.05 $\mu\text{g/g}$. Each symbol represents the simultaneous plasma and rectal tissue concentrations of an individual macaque.

well tolerated and self-limited (19, 20). Although both oral and parenteral formulations of GSK744 continue to be evaluated in additional clinical trials, the safety data to date support further clinical development.

The goal of this work was to evaluate GSK744 LA as a PrEP agent in macaques to establish proof of concept. In anticipation of performing a low-dose intrarectal (IR) challenge experiment, we first conducted a PK study to identify a dosing regimen that could achieve clinically relevant plasma concentrations. Indian rhesus macaques (*Macaca mulatta*) were injected intramuscularly (IM) at two time points 4 weeks apart with 10 mg/kg ($n = 8$) or 30 mg/kg ($n = 4$; two injections of 15 mg/kg) of GSK744 LA, but the mean plasma GSK744 concentrations (Fig. 1A) were not always above $4\times$ PAIC₉₀, a level found to be important for the therapeutic effect in infected patients (18). A single intramuscular dose of 200, 400, or 800 mg of GSK744 LA in healthy volunteers revealed dose-proportional increases in drug exposure [AUC_(0–∞)] (Fig. 1B) (8, 21). The 800-mg dose of GSK744 LA resulted in sustained mean plasma concentrations $>4\times$ PAIC₉₀ for 16 weeks (Fig. 1B). Because of injection volume limits, the 800-mg dose was administered as a split injection (two of 400 mg), resulting in a supraproportional maximum concentration (C_{max}) compared with the 200- and 400-mg doses. This finding suggested a faster release of GSK744 into the circulation as a consequence of dose splitting. However, only a dose-proportional increase in C_{max} was observed when the dose was split in macaques. The elimination $t_{1/2}$ of GSK744 LA was much shorter in macaques, 3 to 12 days, compared with humans, which is often the case for drugs in smaller mammals (22). The faster clearance of GSK744 in macaques required that a higher dose of GSK744 LA be used to maintain clinically relevant plasma concentrations

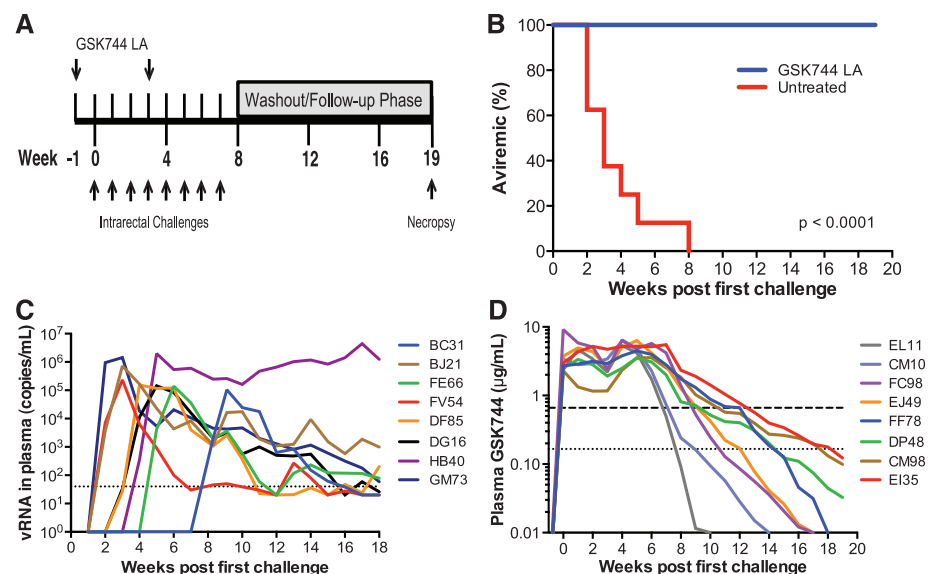


Fig. 2. Monthly injection of GSK744 LA protects macaques against repeated SHIV exposure. (A) Study design. Eight male macaques were injected IM in the quadriceps with 50 mg/kg (four of 12.5 mg/kg) of GSK744 LA at two time points, week -1 and 3. An additional eight male macaques were untreated and served as controls. All animals were challenged IR each week with 50 TCID₅₀ of SHIV162P3 for up to eight exposures or until infection occurred. GSK744 LA-treated macaques were necropsied at week 19 for evaluation of proviral DNA in multiple tissues. (B) Kaplan-Meier plot of treated and untreated macaques remaining aviremic after serial SHIV challenges. (C) Viral loads of individual untreated control macaques. Dotted line represents the LOQ, >40 SHIV RNA copies per milliliter of plasma. (D) Plasma GSK744 concentrations in individual macaques throughout the course of study. Dotted and dashed horizontal lines represent $1\times$ and $4\times$ PAIC₉₀, respectively. LOQ >0.01 $\mu\text{g/mL}$.

throughout the dosing interval in the virus prevention study.

To understand drug distribution to the site of virus inoculation, we determined the levels of GSK744 in rectal biopsy tissues as compared with those observed in the plasma of macaques treated with 10 or 30 mg/kg of GSK744 LA. As expected, higher plasma concentrations correlated with higher tissue concentrations (Fig. 1C). The

ratio of mean rectal tissue to plasma concentrations (T:P) was 0.21 (range from 0.08 to 0.54). The animals treated with GSK744 LA at 30 mg/kg were necropsied 3 weeks after the second dose, and various tissues were analyzed for GSK744 (table S1). The drug was consistently identified in various regions of the gastrointestinal tract (colon, ileum, jejunum, and duodenum) with T:P similar to that observed for rectal tissue. GSK744

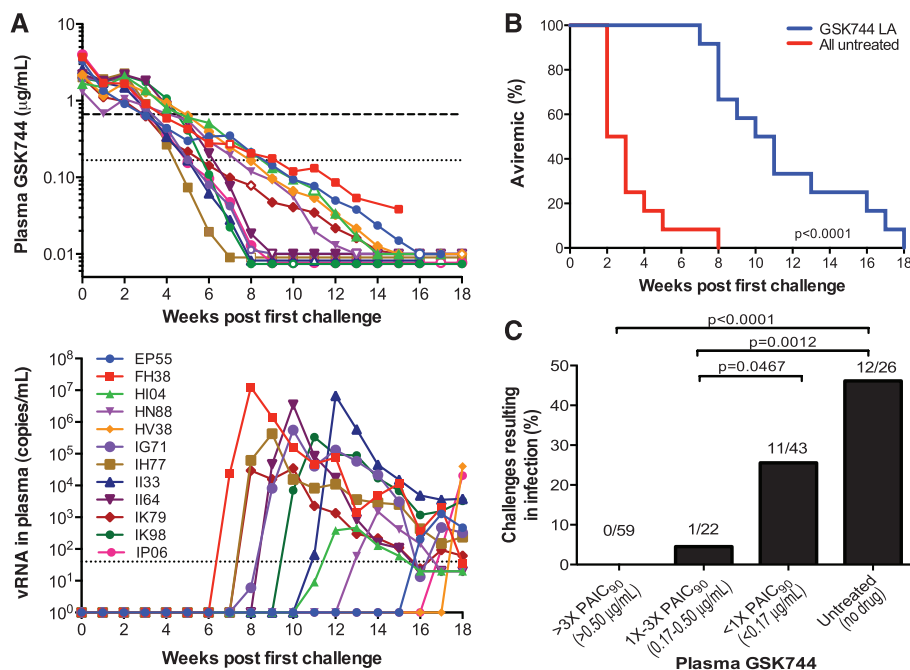


Fig. 3. Plasma GSK744 levels that protected against repeated SHIV exposures. Twelve male macaques were injected IM with GSK744 LA at 50 mg/kg (four of 12.5 mg/kg) 1 week before the first virus exposure. Four macaques remained untreated as controls. All animals were challenged IR each week with 50 TCID₅₀ of SHIV162P3 until infection was confirmed. (A) (Top) Plasma GSK744 concentrations from individual macaques. Open symbols correspond to first detection of viral RNA. Dotted and dashed horizontal lines represent 1x and 4x PAIC₉₀, respectively. LOQ > 0.01 µg/mL. (Bottom) Viral loads of GSK744 LA-treated macaques. Dotted line represents the LOQ at >40 SHIV RNA copies per milliliter of plasma. (B) Kaplan-Meier plot demonstrating the delay of infection in GSK744 LA-treated macaques ($n = 12$) relative to untreated control macaques ($n = 12$; 4 from the current experiment and 8 from the first experiment). (C) Rate of SHIV infection within various ranges of GSK744 concentrations in plasma. The numbers above the bars represent the number of infections/number of challenges within a plasma concentration range. Plasma concentrations $>3 \times \text{PAIC}_{90}$ yielded 100% protection, whereas concentrations $>1 \times \text{PAIC}_{90}$ resulted in an infection rate of 1.2% (0/59 and 1/22). Compared with a 46.2% (12/26) infection rate in the placebo macaques, one can calculate a protective efficacy of ~97% in macaques with drug concentrations $>1 \times \text{PAIC}_{90}$.

was detected in all lymphoid tissues analyzed, including cervical, inguinal, mesenteric, and axillary lymph nodes as well as tonsil and spleen. The highest GSK744 concentration in tissue was found in the liver, which may be due to the uptake of nanoparticles by Kupffer cells (23). The muscle tissues at injection sites had the lowest GSK744 concentration, suggesting that the drug was largely released by the time of necropsy.

To evaluate the efficacy of GSK744 LA as PrEP, we used a repeat low-dose rectal challenge macaque model (24–28), which was developed to more closely mimic HIV-1 exposures in humans. Previously, using the same model, oral FTC/TDF demonstrated protection as observed in the iPrEx study among adherent volunteers (26). The earlier PK results from 10 and 30 mg/kg of GSK744 LA dosing indicated that a dose of 50 mg/kg would likely maintain plasma concentrations comparable to those in GSK744 LA-treated humans throughout the dosing period. Eight macaques were therefore injected IM with 50 mg/kg of GSK744 LA at two time points 4 weeks apart, starting 1 week before the first virus exposure (Fig. 2A), and an additional eight macaques remained untreated

as controls. Both groups of macaques were challenged by nontraumatic inoculation of 1 ml of SHIV162P3 (50 TCID₅₀) into the rectal vault through a sterile gastric feeding tube. The macaques were similarly challenged weekly for up to eight challenges or until infection was confirmed by real-time reverse transcription polymerase chain reaction amplification of viral *gag* sequences from plasma. GSK744 LA-treated animals remained aviremic as assessed by weekly measurements during the challenge period as well as the wash-out phase (Fig. 2B). In contrast, untreated macaques became infected during the challenge period, requiring a median of two challenges (range from 1 to 7; Fig. 2, B and C). GSK744 LA-treated macaques had a 28.2-fold [hazard ratio, 95% confidence interval (CI) 5.8, 136.8] lower risk of infection compared with untreated macaques ($P < 0.0001$, log-rank test).

As expected, dosing macaques with GSK744 LA at 50 mg/kg yielded mean drug exposure above 4x PAIC₉₀ (Fig. 1A) throughout the period of virus challenge. The interanimal variability in PK (Fig. 2D) was anticipated on the basis of similar results observed in humans. All macaques,

except EL11, maintained plasma drug concentrations $>4 \times \text{PAIC}_{90}$ throughout the challenge period. GSK744 concentrations in the plasma of EL11 fell to 0.50 µg/mL for the last challenge (week 7), whereas the other macaques sustained plasma drug concentrations $>4 \times \text{PAIC}_{90}$ through weeks 7 to 12. The $t_{1/2}$ of GSK744 LA in monkeys was indeed shorter, ranging from 5 to 12 days. Nevertheless, the mean plasma GSK744 concentrations achieved in the protected macaques closely parallel plasma concentrations measured in humans administered a single, 800-mg intramuscular dose of GSK744 LA (fig. S1).

SHIV-specific antibodies in plasma were detected by means of an enzyme immunoassay in all untreated controls 1 to 3 weeks after viral RNA detection but were not detected in any GSK744 LA-treated macaques throughout the study (table S2). Proviral DNA was not identified in PBMCs from any drug-treated macaque throughout the study (table S2). Further virologic studies were performed to verify that the GSK744 LA-treated macaques were not locally infected despite lacking evidence of systemic viral dissemination. The macaques were necropsied 19 weeks after the first challenge, and tissues at the sites of virus inoculation and draining lymph nodes were analyzed for the presence of proviral DNA. To increase the sensitivity of the assay, we enriched rectum and colon tissues for mucosal mononuclear cells before analyzing the tissues for proviral DNA. No proviral DNA was detected in any of the samples, with the exception of the rectal mucosal mononuclear cells from EL11, where a positive signal below the limit of quantitation was observed (2 positive out of 20 wells assayed) (table S2). Although proviral DNA was detected at an extremely low level, a productive systemic infection was never established during the 9 weeks of follow-up when plasma GSK744 concentrations were below the limit of detection.

A follow-up experiment was then performed to determine the minimal drug level that affords protection against repeated low-dose IR SHIV challenges. Twelve macaques were injected IM with 50 mg/kg of GSK744 LA once 1 week before the first virus exposure. All macaques were challenged IR each week with 50 TCID₅₀ of SHIV162P3 until systemic infection was detected. An additional four macaques remained untreated as controls. In anticipation of the long duration of the experiment, one control macaque began the series of virus challenges every 4 weeks. All control macaques became infected quickly, after 1 or 2 challenges, as evidenced by rising viremia (fig. S2). GSK744 LA-treated macaques remained aviremic throughout the initial phase of SHIV challenges, but as the plasma drug concentrations declined (Fig. 3A, top) they became infected, gradually and successively (Fig. 3A, bottom). Overall, the treated animals were infected after 6 to 17 (median of 10) virus challenges compared with 1 to 7 challenges (median

of 2, based on data from untreated macaques from both experiments) for untreated controls (Fig. 3B). Thus, a single dose of GSK744 LA delayed infection by 5 to 10 challenges (median of 8) compared with untreated macaques. Assuming a 2-week eclipse phase between the start of infection and detection of viremia, we calculated plasma GSK744 levels that coincided with the start of SHIV infection (fig. S3). All infections occurred when the plasma drug concentrations were below 0.50 $\mu\text{g/ml}$, or $\sim 3\times$ PAIC₉₀. A more detailed analysis was then carried out to calculate the percent of virus challenges that resulted in infection within various ranges of plasma GSK744 concentrations (Fig. 3C). The 12 GSK744 LA-treated macaques were collectively exposed to 59 SHIV challenges at plasma concentrations $>3\times$ PAIC₉₀, and none resulted in infection. One of 22 challenges at plasma concentrations between $1\times$ and $3\times$ PAIC₉₀ led to infection. As plasma GSK744 concentrations decreased below $1\times$ PAIC₉₀, 11 infections resulted from 43 virus challenges, yielding an infection rate of 25.6%. This value was lower than the 46.2% (12/26) infection rate observed in untreated macaques, but the difference was not statistically significant ($P = 0.11$, Fisher's exact test). On the basis of the analysis shown in Fig. 3C, we determined that plasma GSK744 concentrations $>3\times$ PAIC₉₀ conferred 100% protection, whereas concentrations $\geq 1\times$ PAIC₉₀ conferred $\sim 97\%$ protection (see legend for calculation). No signature integrase resistance-conferring mutations were identified in breakthrough viruses (table S3). This second experiment not only defined the correlate of protection but also confirmed the results of the first experiment by showing that sufficiently high but clinically achievable GSK744 concentrations could effectively protect all macaques from repeated IR SHIV challenges.

GSK744 LA appears to be a promising next-generation PrEP agent that has afforded high-level protection against repeated IR SHIV challenges in rhesus macaques (Figs. 2B and 3B). Plasma drug levels $>3\times$ PAIC₉₀ provided 100% protection, whereas levels $\geq 1\times$ PAIC₉₀ provided $\sim 97\%$ protection (Fig. 3C). These plasma concentrations can be readily achieved in humans with quarterly 800-mg IM injections of GSK744 LA (Fig. 1B and fig. S1). Given that the half-life of GSK744 LA is 3 to 12 days in macaques whereas the half-life is 21 to 50 days in humans, we anticipate a longer-lasting protective effect in humans. Nevertheless, the proof will have to be demonstrated in future clinical trials. Optimistically, toxicity issues notwithstanding, GSK744 LA has the potential to achieve in preventing HIV-1 infection what a long-acting contraceptive has achieved in preventing unintended pregnancies (29). It also stands to reason that the protective efficacy of GSK744 LA could approximate the high efficacy ($>90\%$) observed in high-risk participants who were most adherent to daily oral FTC/TDF as PrEP (3, 6). These considerations,

coupled with a favorable drug safety profile, have placed GSK744 LA on track for phase 2 (safety) clinical trials as well as a phase 3 (efficacy) study in men who have sex with men. Follow-up macaque experiments to protect against intra-vaginal and intravenous SHIV or SIV challenges could establish the proof of concept to move into efficacy trials in other populations at high risk for HIV-1 infection.

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Supplementary Materials

www.sciencemag.org/content/343/6175/1151/suppl/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S3
References (30–33)

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Altitudinal Changes in Malaria Incidence in Highlands of Ethiopia and Colombia

A. S. Siraj,^{1*} M. Santos-Vega,^{2*} M. J. Bouma,³ D. Yadeta,⁴ D. Ruiz Carrascal,^{5,6} M. Pascual^{2,7†}

The impact of global warming on insect-borne diseases and on highland malaria in particular remains controversial. Temperature is known to influence transmission intensity through its effects on the population growth of the mosquito vector and on pathogen development within the vector. Spatiotemporal data at a regional scale in highlands of Colombia and Ethiopia supplied an opportunity to examine how the spatial distribution of the disease changes with the interannual variability of temperature. We provide evidence for an increase in the altitude of malaria distribution in warmer years, which implies that climate change will, without mitigation, result in an increase of the malaria burden in the densely populated highlands of Africa and South America.

The impact of warming temperatures on highland malaria remains a subject of debate (1–7). Malaria is a multifactorial disease, because the etiological agent has a complex life cycle requiring an insect vector, and the factors that regulate its distribution and abundance are diverse and complex (8). Despite the expectation that global warming should lead to an increase

in the altitudinal range of malaria, empirical evidence for this phenomenon is lacking, and the attribution of trends to specific factors remains difficult because of multiple drivers, including drug resistance, land-use change, human migrations, and access to health facilities (9, 10). An increasing altitudinal range implies the potential for an increased burden of malaria with climate change,

especially for countries of East Africa and South America with densely populated highlands that have historically provided havens from this devastating disease (17). Colder temperatures at higher altitude in these tropical latitudes slow down and even halt the development of the parasite inside the mosquito vector, decrease the rate of reproduction, and reduce the biting rate of the vector, minimizing, if not preventing, transmission (8).

In recent decades (1970–2000), pronounced increases in malaria incidence have been documented at several locations in Africa (1, 2, 12) for which long-term temporal records exist and which precede the greater intervention efforts of the past decade (13). Disease trends, no less than climate warming trends in earlier studies (6, 14–16), generate debate (5), but what has been missing is analysis of spatiotemporal records. Although range shifts have been documented with empirical evidence for the distribution of several plant and animal species (17, 18), similar patterns in vector-borne illnesses and pathogens of humans remain largely unexplored. An increasing incidence with altitudinal elevation in warmer years would be a clear signal of the response of highland malaria to changes in climate.

Hence, we looked for evidence of a changing spatial distribution of malaria with varying temperature for over a decade in highland regions of northwest Colombia and central Ethiopia (fig. S1). To do so, we considered temperature variability at interannual time scales rather than long-term trends. Temporal associations between malaria and climate variability have been described for endemic regions of Colombia (19, 20) and for epidemic regions in the highlands of Ethiopia and other East African countries (2, 21, 22). Thus, we specifically asked how temperature variability influences the spatial distribution of disease incidence along altitudinal gradients.

The records we used consisted of monthly *Plasmodium falciparum* cases for 124 municipalities in the Antioquia region in western Colombia for 1990–2005 and for 159 administrative units, known as kebeles, in the Debre Zeit area of central Ethiopia for 1993–2005 (23) (figs. S1 and S2). When we clustered kebeles or municipalities on the basis of similar spatiotemporal dynamics (23), the resulting communities exhibited significant dif-

ferences in altitude (Fig. 1). The importance of altitude to the spatial distribution of the disease was reflected in the observation of a decrease in malaria incidence as altitude increased, as expected from the concomitant decrease in temperature (fig. S6). This classic feature of the epidemiology of malaria was first reported in the 19th century (24, 25).

Because the absolute number of malaria cases from one year to the next can vary in relation to several demographic and environmental factors, we wanted to compare the spatial distribution of cases across years in a way that is independent of the temporal variation, including of long-term trends, in the total burden of disease. Hence, we built cumulative distribution curves for yearly cases during the epidemic season across the elevation gradient (23). The altitude corresponding to the median of this cumulative distribution is the “median altitude.” The median altitude changed with time to reflect the movement of the distribution up or down the altitudinal gradient. Figure 2, A and D, illustrate these patterns for two given years and shows that the median altitude for case distribution changed across years

as a function of the average temperature in a critical period preceding the epidemic season (Fig. 2 and fig. S5). Thus, the median altitude for case distribution increased with mean temperature (Fig. 2, B and E), and malaria cases occurred at higher elevations in warmer years, notably in 1997 and 2002, in both regions (Fig. 2, C and F). This synchrony between continents (26) may be related to above-normal global temperatures that accompany El Niño events. Similar results were obtained for quantiles of the cumulative distribution above the median (fig. S7), which showed that the change in the spatial distribution did not concentrate in its center but affected its whole upper part (see also fig. S8).

Vector control by spraying residual insecticides was scaled-up after the 2002–2004 epidemic in Ethiopia and has resulted, in combination with more effective medication, in a considerable reduction of cases (13). Because the limited insecticide-spraying operations before 2004 may have been restricted to the lower-altitude contours that are typically considered epidemic-prone, we examined whether selective spraying of lower altitudes could account for the altitudinal shifts seen

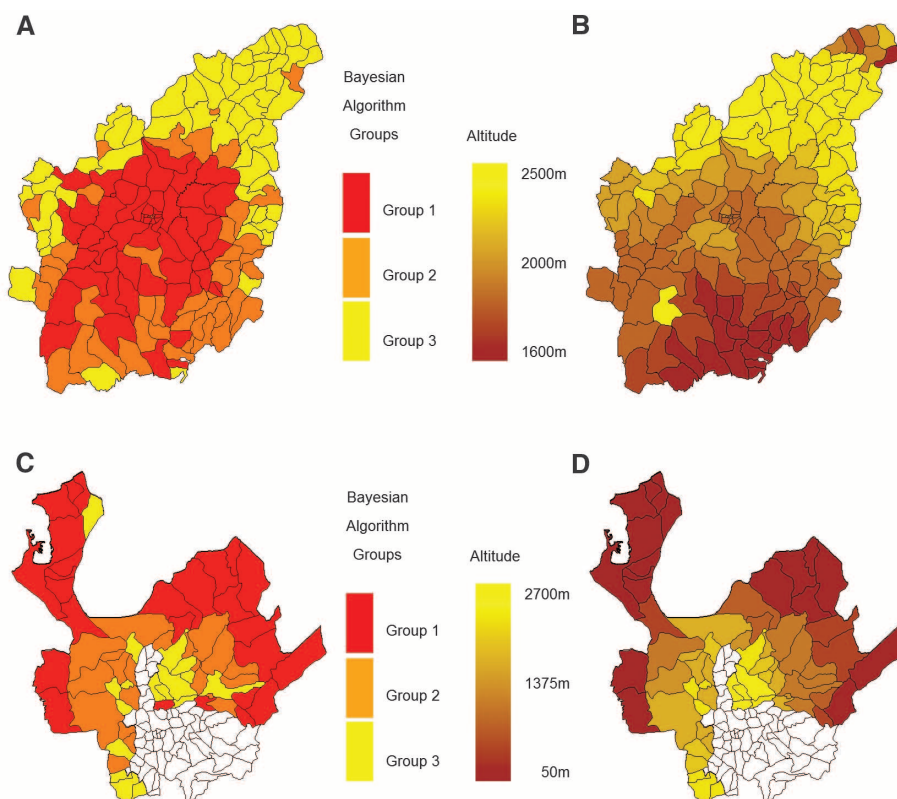


Fig. 1. Geographical areas based on similar temporal dynamics of malaria cases correspond to different elevations. (A and C) The three different sets of administrative units categorized by temporal patterns of monthly cases in the study regions in Ethiopia and Colombia, respectively. These sets were obtained with a Bayesian grouping algorithm that identified locations based on similar temporal dynamics using a nonparametric Markov transition model (23). (B and D) The corresponding elevation maps, with elevation weighted by the population sizes within each location. The identified sets correspond to significant differences in altitude [analysis of variance (ANOVA), $P < 0.01$ for (A) and (C), eastern region, and $P < 0.001$ for (C), western region]. Municipalities not included in the analysis are shown in white; these correspond to locations in Colombia with either incomplete time series or no malaria (above 2600 m).

¹Department of Geography and the Environment, University of Denver, 235 Boettcher West, 2050 East Iliff Avenue Denver, CO 80208-0710, USA. ²Department of Ecology and Evolutionary Biology, University of Michigan, 2019 Kraus Natural Sciences Building, 830 North University, Ann Arbor, MI 48109-1048, USA. ³London School of Hygiene and Tropical Medicine, University of London, London WC1E 7HT, UK. ⁴Oromia Regional Health Bureau, Post Office Box 24341, Addis Ababa, Ethiopia. ⁵International Research Institute for Climate and Society, Columbia University in the City of New York, Lamont-Doherty Earth Observatory Post Office Box 1000, 61 Route 9W, Monell Building, Palisades, NY 10964-1000, USA. ⁶Escuela de Ingeniería de Antioquia, km 02+200 Vía al Aeropuerto José María Córdova, Envigado, Antioquia, Colombia. ⁷Howard Hughes Medical Institute, Chevy Chase, MD 20815-6789, USA.

*These authors contributed equally to this work.

†Corresponding author. E-mail: pascual@umich.edu

in the malaria case data. Specifically, because our information from Debre Zeit specifies the kebeles that have received spraying since 1994, we were able to exclude these locations from the analysis and to confirm that our results on the median altitude for the case distribution remain unchanged (fig. S9).

Spraying operations in Colombia were scaled back when eradication efforts ceased, particularly after 1993, when low-cost DDT was banned. Between 2000 and 2007, Colombia reported no insecticidal residual spraying (IRS) operations (27), and vector control is not likely to have skewed malaria's altitudinal distribution in our Colombian study area. Rainfall and the variation of rainfall between years are also not likely to have biased our results. Over the altitude ranges in both regions, monthly rainfall during the wet season exceeds 80 mm, which is considered optimally suitable for malaria transmission (28). Furthermore, over these ranges, rainfall increases with

altitude in Ethiopia but decreases in Colombia (29). Despite differences in rainfall regimes and in local vectors and vector ecology, which give rise to noticeable differences between the seasonality of malaria in both regions (fig. S3), the comparable response in both countries of malaria distribution to temperature variations highlights the importance of this climate parameter in highland areas.

To analyze the variation in incidence with temperature and altitude, we fitted a negative binomial regression to the monthly cases (23). Covariates included season, altitude, and linearly de-trended temperature (lagged by 3 months), where the latter represented a regional mean value. Model selection [based on the Akaike information criterion (AIC)] was used to compare models with different numbers of covariates and their interactions. For both regions, the best model included temperature, seasons, and altitude, as well as a mix of the two-way and three-way interactions

between these covariates (Table 1). More importantly, the best statistical model showed a significant positive effect of mean temperature on the logarithm of malaria cases for both regions. This effect corresponded to a percent change in cases between 35 and 64% per 1°C for kebeles in Ethiopia at the peak of the malaria season (fig. S10 A). In Colombia, this rate ranged between approximately 10 and 80% from the highest to the lowest municipalities (fig. S10 B).

Our results showed that despite being on two different continents, in these two highland regions, increases in temperature across years extended the spatial distribution of malaria cases to higher elevations. The implication is that global warming will increase the risk of contracting highland malaria in the future. The rapid climate variations associated with the pronounced topographic heterogeneities of highland regions are poorly captured by the coarse resolution of global circulation models and their future projections (30), and the

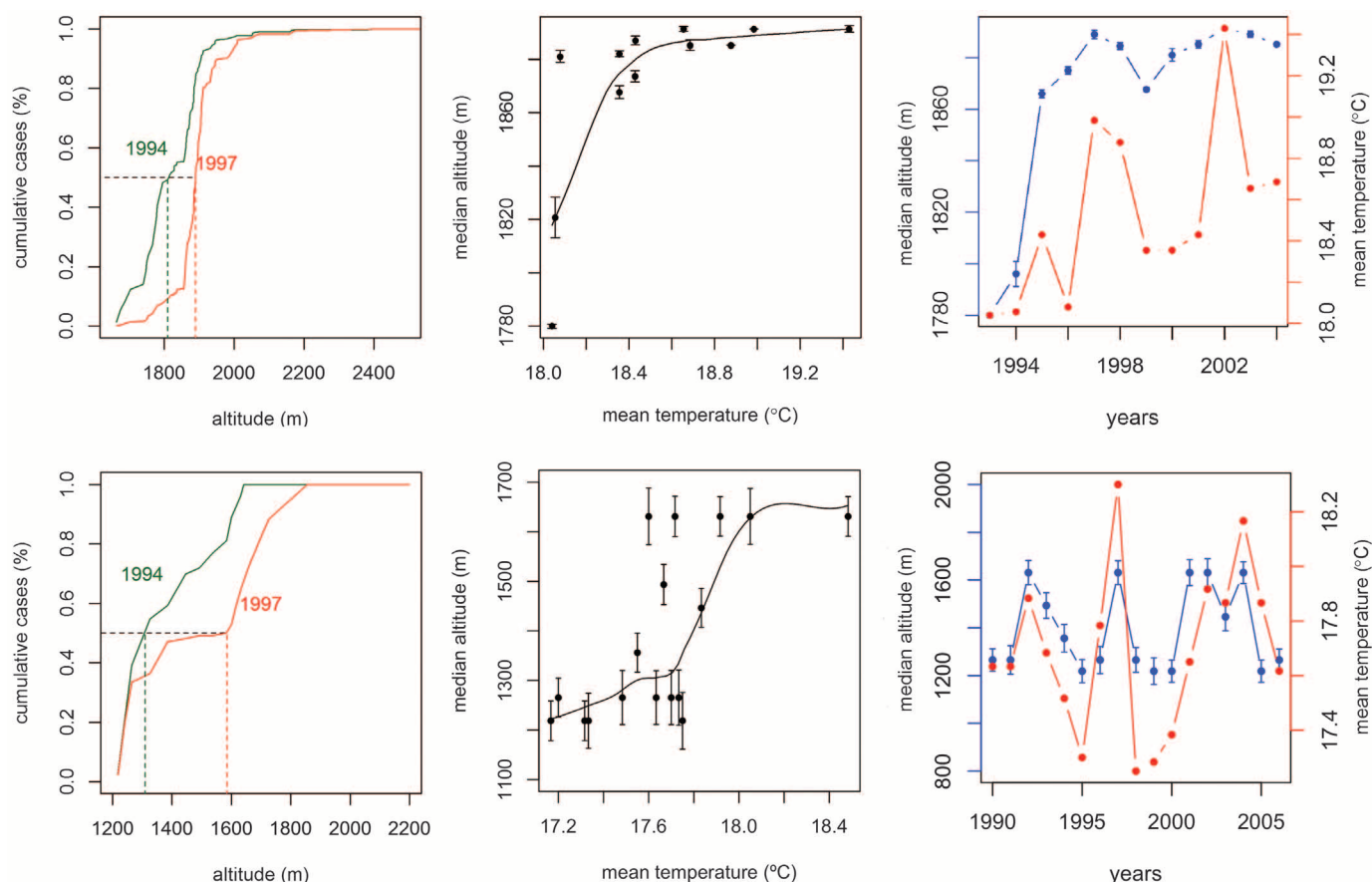


Fig. 2. Changes in altitudinal distribution of malaria cases with mean temperature across years. Altitudinal cumulative distributions of cases for Ethiopia (top row) and Colombia, western region (bottom row) are shown as a function of time, as well as mean temperatures preceding the transmission season. (A and D) The altitudinal cumulative curves generated with the incidence data in two given years, together with the location of the 50th percentile and its corresponding altitude. By definition, this is the altitude at which 50% of the cases occur below and 50%, above in the altitudinal gradient. A shift of this cumulative curve to the right indicates that more malaria cases have occurred at higher altitudes in a given year. This does not

mean the number of cases has increased from 1994 to 1997 but that the distribution of the disease has moved toward a higher elevation. (B and E) The corresponding scatter plots of the median altitude against these temperatures, demonstrating a movement of the distribution to higher altitudes in warmer years for the two highland regions. (C and F) show the yearly variation in the median altitude of cases (blue line), together with the mean temperatures in the critical 4-month window for the two regions (red line) (fig. S5). Uncertainty in the median value is estimated by bootstrap resampling (23) and is shown as 1 SD in the plots. (The eastern region of Colombia exhibits similar patterns to those shown here for the western region.)

intricacies of mountain climates may complicate local climate change predictions. Moreover, highlands tend to be poorly covered by the global network of meteorological stations (29); however, because of the potential attribution of rising malaria to climate warming, station temperature records in East African highlands have received more attention (1, 14, 15). In particular, at the Debre Zeit station, significant trends in rising day and night temperatures are uncontroversial (31). In Colombia, highland areas are warming faster than the surrounding lowlands; a pattern reflected in weather station data and previously suggested

by atmosphere-ocean coupled general circulation models (32, 33).

The spread of chloroquine resistance has been proposed as the main reason behind the increasing trends in malaria time series in the Kenyan highlands (9). Our retrospective data sets can be extended backward in time to the early 1980s (albeit with no spatially explicit information) for the whole aggregated region of Debre Zeit in Ethiopia and for a single municipality, Anori, in Colombia, and for both parasites, *P. falciparum* and *P. vivax* (Fig. 3 and fig. S4) (23). Although drug resistance to chloroquine almost exclusive-

ly applies to *P. falciparum* [and this was also the case in the Debre Zeit region (34)], the longer time series of cases show similar increasing trends from the 1980s to the 1990s for both parasites in both countries. This increase occurred before the intensification of vector control and treatment of the past decade led to lower levels of malaria (13).

Climate change appears to have already influenced the burden of malaria in these regions. Specifically, the trend for malaria in Ethiopia since the 1980s (Fig. 3) is consistent with the rate of change we would expect from the interannual variation in the spatiotemporal data (23) (fig. S11). This expected change is approximately 2160 cases per degree Celsius over a season (September to December), and the value obtained from fitting linear trends to both the *P. falciparum* and temperature time series is of similar magnitude: 2310 cases. Consistent values were also obtained for Colombia (23) (fig. S11), although these values are smaller because the long time series concern a single municipality with a small population, and not the whole region studied. This means that the change expected from increasing temperatures alone can account for the retrospective temporal trend in cases observed in the recent past at both locations.

It has been argued that the global effect of climate change on malaria will be negligible as compared with the potential impact of intervention and improved socioeconomic conditions (35). However, in the East African highlands a strong temperature-determined malaria lapse rate persists (7). Elevated potential transmission intensity, driven by warmer temperatures, will require even greater control efforts, especially in countries such as Ethiopia with large populations at high elevation. In the altitude range of the Debre Zeit sector, between 1600 and 2400 m, approximately 37 million people live in rural areas at risk of malaria, which corresponds to 43% of Ethiopia's total population. Based on Ethiopia's current population size and the malaria lapse rate from pre-control mass survey prevalence data since the 1930s (36), we had previously estimated that a 1° rise in temperature would result, without mitigation, in an additional 410,000 infections per year as a result of the territorial extension of malaria (11). An additional 2.8 million annual infections would occur in the population younger than 15 years of age living at altitudes where the disease would intensify and where adults already have a degree of immunity (11). These estimates were based on the assumption of a temperature-dependent shift of the disease to higher altitudes (37, 38). By supporting this assumption, our findings here underscore the size of the problem and emphasize the need for sustained intervention efforts in these regions, especially in Africa, where more highly anthropophilic vectors preclude extrapolation from the earlier and easier victories made against malaria at the fringes of temperate climates (7). Fortunately, the control of malaria at the edge of its

Table 1. Parameter estimates for the negative binomial regression model.

Covariates	Coefficient (standard error)	
	Ethiopia	Colombia (western region)
(Intercept)	-9.3348 (0.0402)***	-37.1670 (5.2322)***
Season factor	9.6623 (0.3509)***	23.8540 (5.7220)***
Temperature	0.1880 (0.0316)***	1.3730 (0.2740)***
Altitude	-0.0037 (0.0001)***	-0.0004 (0.0010)
Temperature:altitude	-0.0002 (0.0001)***	-0.0002 (0.0001)**
Temperature:season	0.8437 (0.3919)*	-11.7047 (3.0020)***
Season:altitude	-0.0091 (0.0010)***	
Season:temperature:altitude		0.0003 (0.0012)***

***P value < 0.001.

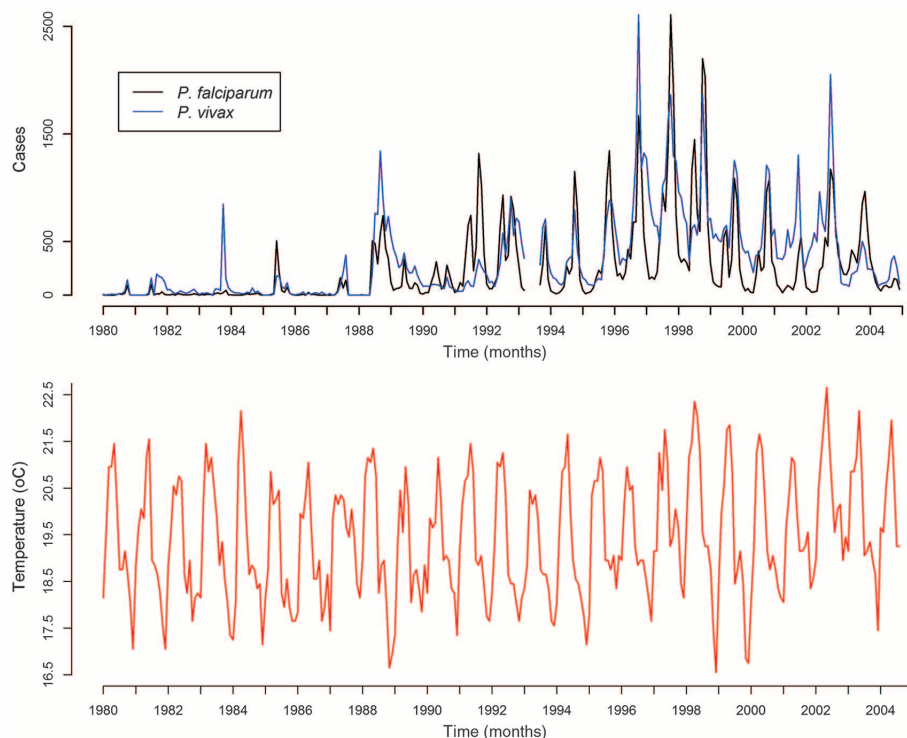


Fig. 3. Temporal trends in temperature and malaria cases for the whole region of Debre Zeit (Ethiopia). The malaria cases for *P. falciparum* and *P. vivax* (top panel) are shown with the corresponding mean temperatures (bottom panel). Both *P. falciparum* and *P. vivax* cases exhibit an increasing trend from the 1980s to the end of the 1990s. So does mean temperature by 1°C in 378 months (estimated as a linear trend). An increase of 1°C corresponds to an additional 2166 cases during the main transmission season (from September to December). This malaria increase over time is consistent with what is expected from the trend in temperature, given the described change in cases with the interannual variation of temperature over a shorter time period (23) (fig. S11). A long-term trend in cases is also shown for Colombia but for a single municipality (fig. S4).

altitudinal distribution is considerably easier than in highly endemic, lower-elevation regions. Despite Africa's exceptionally efficient vectors, an early eradication pilot scheme in highland regions shows that malaria transmission at altitudes in excess of 1130 m (3700 feet) can be fully interrupted (39). Public health policies should therefore be formulated that mitigate the effects of climate change on malaria in highland regions, including those policies that extend and sustain the network of diagnostic and monitoring facilities. These measures should further contribute to the early warning of epidemics and assist global malaria elimination.

References and Notes

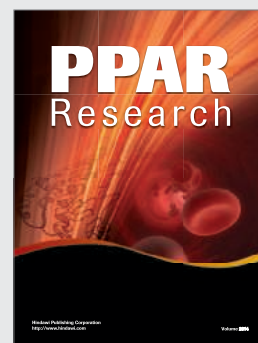
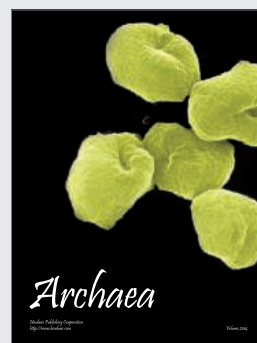
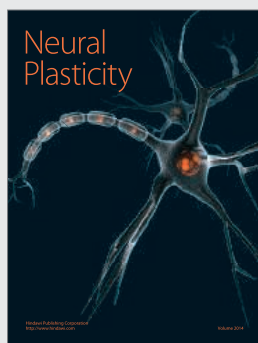
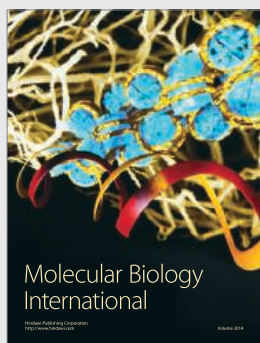
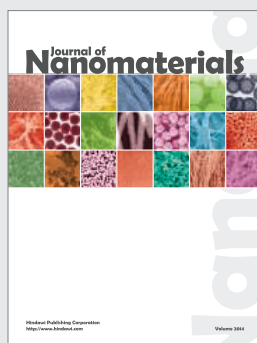
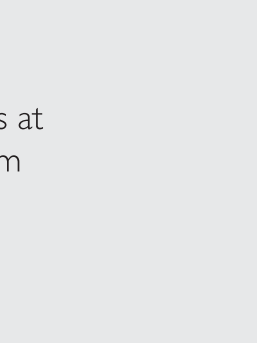
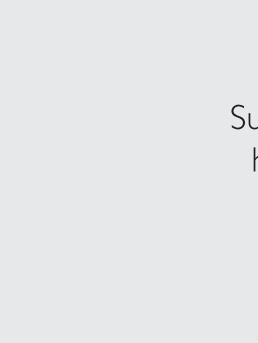
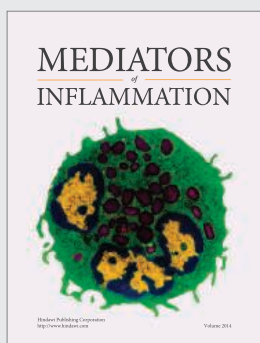
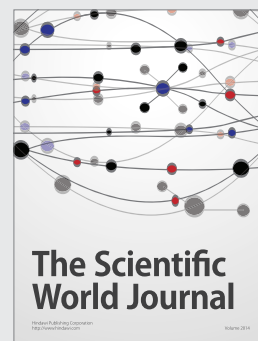
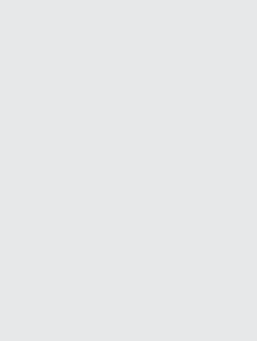
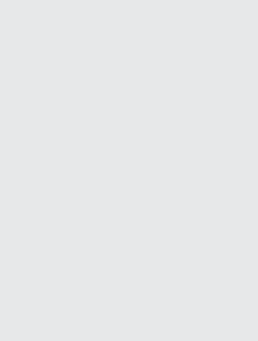
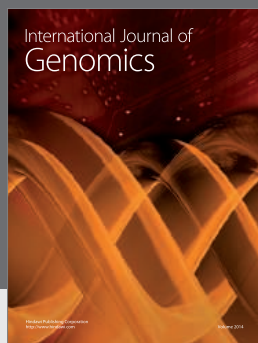
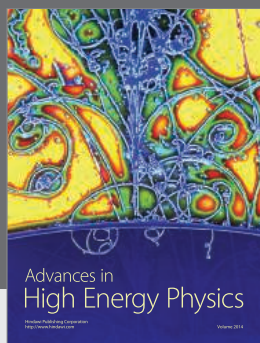
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Supplementary Materials

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Materials and Methods
Figs. S1 to S11
References (40–47)

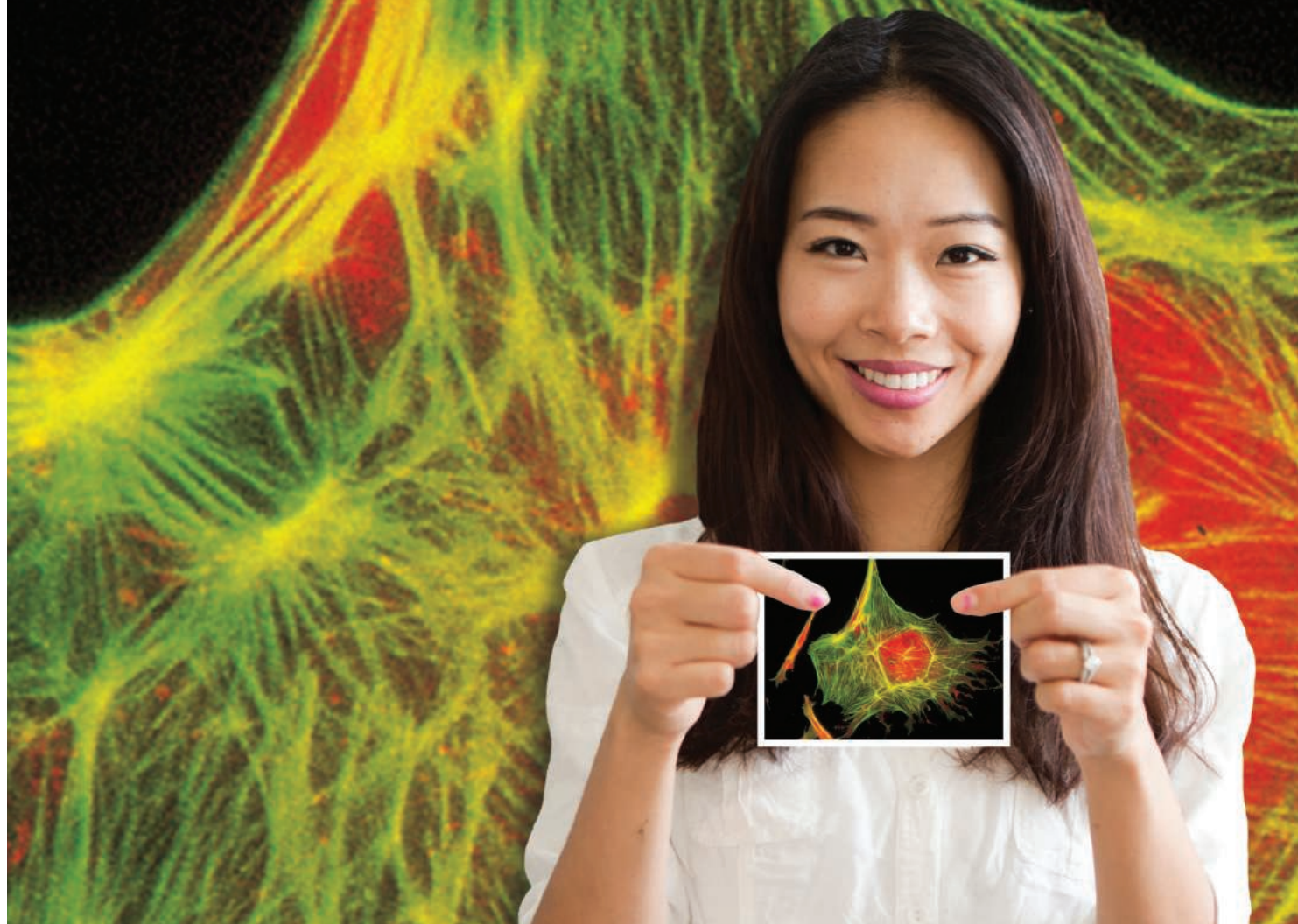
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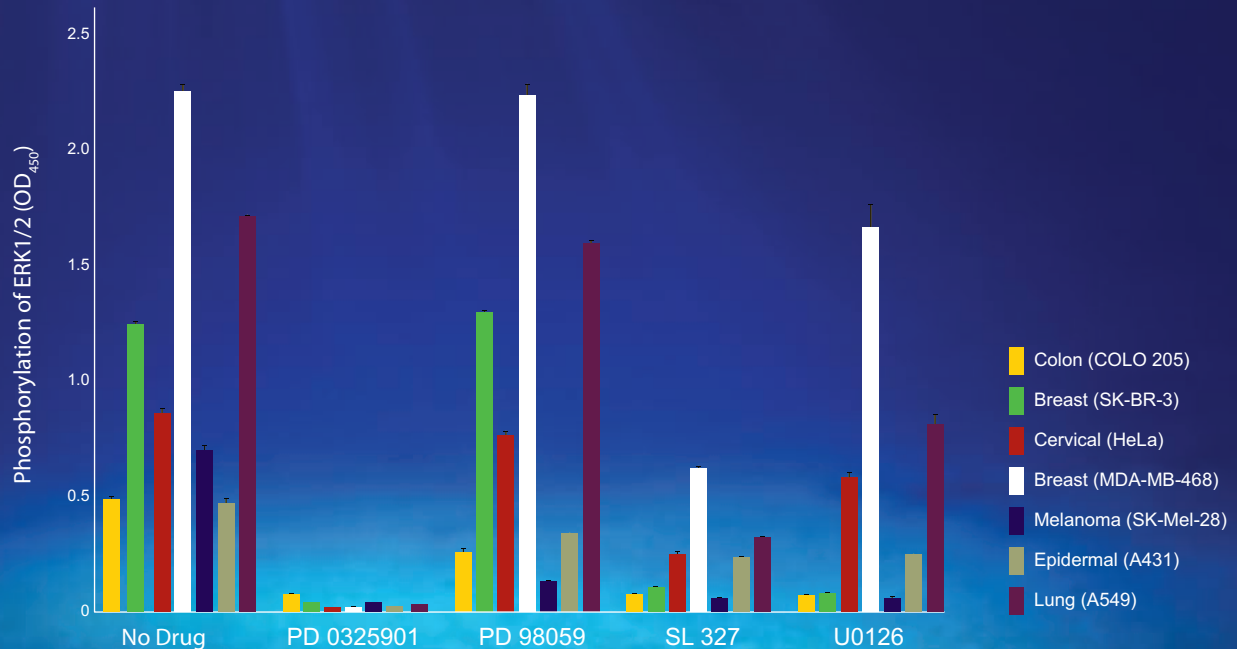
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Director, School of Veterinary Medicine and Biomedical Sciences

The University of Nebraska-Lincoln (UNL) Institute of Agriculture and Natural Resources (IANR) is seeking candidates for the position of Director, School of Veterinary Medicine and Biomedical Sciences (SVMBS) and Associate Dean, Professional Program in Veterinary Medicine (PPVM).

The Director of the SVMBS is directly responsible for oversight of the research, teaching and extension/outreach programs, including management of the Nebraska Veterinary Diagnostic Center, the Professional Program in Veterinary Medicine (PPVM) - a joint DVM program with Iowa State University - and clinical education programs emanating from the Great Plains Veterinary Educational Center (GPVEC). The SVMBS has a tradition of excellence in graduate education and research with an emphasis on infectious diseases, immunology, and neurosciences; the Director will provide leadership and insight for these programs as well. The Director reports to the Deans of the ARD, CASNR, and Extension at UNL. As the Associate Dean of the PPVM the successful candidate also reports to the Dean of the Iowa State University College of Veterinary Medicine. The Associate Dean is responsible for on-going coordination of the professional program with students located at the Iowa State University College of Veterinary Medicine and the UNL SVMBS. Visit the SVMBS web page for additional information at <http://vbms.unl.edu/>

Candidates must possess (1) Doctor of Veterinary Medicine (D.V.M) or equivalent and advanced degree or Board Certification in a recognized veterinary specialty; (2) a record of scholarly achievements and qualifications for appointment as a tenured, full professor in the School of Veterinary Medicine and Biomedical Sciences; and (3) a documented record of collaborative leadership experience in higher education, business, or non-profit organizations. Earned Doctor of Philosophy (Ph.D.) or Doctor of Science (D.Sc.) degree in veterinary science or closely related field, excellent communication skills and excellent interpersonal skills are preferred.

Nebraska ranks #4 in the US in total livestock receipts and livestock products account for about 2/3 of Nebraska's farm income. Historically, Nebraska has ranked #1 in commercial red meat production, #1 in commercial cattle slaughter, and #2 in cattle and calves. In February 2014, Nebraska became the #1 cattle feeding state in the nation.

To view the details and make application, go to <http://employment.unl.edu>. Search for position #F_140012. Click on "Apply to this job." Complete the application and attach a letter of interest, curriculum vitae, contact information for three professional references, and a one-page statement of administrative philosophy (Other). Review of Applications will begin on April 15, 2014, and continue until the position is filled or the search is closed.

The University of Nebraska has an active National Science Foundation ADVANCE gender equity program and is committed to a pluralistic campus community through Affirmative Action, Equal Opportunity, work-life balance, and dual careers.



**The Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine invites applications for**

**Assistant Professor Faculty Position in Infectious
Disease Research**

The Department of Microbiology, Immunology and Cell Biology seeks a distinguished scientist for an Assistant Professor position to further its scientific exploration and discovery that targets prevention and treatment of infectious disease. The selected candidate will exhibit potential for significantly advancing discoveries in bacterial pathogenesis. He/she will hold a primary faculty appointment in the Department of Microbiology, Immunology and Cell Biology in the WVU School of Medicine.

This recruitment provides an exceptional opportunity to participate in robust, interdisciplinary basic and translational research and training programs in a highly collaborative atmosphere. The successful candidate will be a recognized investigator, with a PhD or MD/PhD degree, and a track record of independent research, demonstrated by high quality publications in peer-reviewed journals. Preferably, the selected candidate will bring research funding commensurate with the position to continue the pursuit of a relevant infectious research agenda. The appointed faculty will be provided with laboratory space and start-up funding for conducting infectious disease research and will be expected to contribute to the teaching mission of the department. The WVU Health Sciences Center is opening a newly constructed A/BSL3 facility in the spring of 2014, which will afford the successful candidate with the facilities to conduct research on agents requiring a high-containment laboratory.

Founded in 1867, West Virginia University is 1 of only 11 research-intensive land-grant institutions offering a Health Sciences campus with accredited Schools of Medicine, Dentistry, Nursing, Pharmacy, and Public Health. The WVU Health Science campus is located in close proximity to WVU campuses that house the Eberly College of Arts & Sciences, the Davis College of Agriculture, the College of Business & Economics, the College of Creative Arts, The Benjamin M. Statler College of Engineering & Mineral Resources, the Perley Isaac Reed School of Journalism, and the College of Law. WVU is West Virginia's only comprehensive doctoral-granting institution and is its major research institution, performing research that is sponsored by the receipt of over \$138 million in contracts and grants annually. The Carnegie Foundation for the Advancement of Teaching classifies WVU as a comprehensive doctoral institution with medical programs placing it among only 50 such public and 28 private institutions nationwide.

Nominations, applications (including a cover letter, research statement, vitae, and a list of 3 professional references), expressions of interest, requests for information, or confidential inquiries should be directed (preferably electronically) to:

Rosana Schafer, Ph.D., Chair, Search Committee
c/o Tammy S. Miller (tsmiller@hsc.wvu.edu)

Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine
Morgantown, WV 26506-9177

The position remains open until filled.

**Open Rank Faculty Position in Neural Injury-Related
Inflammatory Disease Research**

The Department of Microbiology, Immunology and Cell Biology (<http://medicine.hsc.wvu.edu/micro/>) seeks a distinguished scientist as tenure-track faculty (rank open) to further its scientific exploration and discovery that targets prevention and treatment of inflammatory disease associated with neural injury or neurodegeneration. The successful applicant will hold a primary faculty appointment in the Department of Microbiology, Immunology and Cell Biology as well as membership in the WVU Center for Neuroscience (<http://www.hsc.wvu.edu/wvucn/>) in the WVU School of Medicine.

This joint recruitment with the Center for Neuroscience provides an exceptional opportunity to participate in robust, interdisciplinary basic and translational research and training programs in a highly collaborative atmosphere. The appointed faculty will be expected to conduct research on CNS inflammatory disease and teach immunology to medical, graduate and undergraduate students. The successful candidate will be a recognized investigator with a PhD or MD/PhD degree and a track record of independent research, demonstrated by high quality publications in peer-reviewed journals and extramural funding commensurate with years of experience. Preferably, the selected candidate will hold active extramural funding in order to integrate rapidly with ongoing research at the intersection of immunology and neuroscience. Dedicated laboratory space and competitive operating funds will be made available.

Founded in 1867, West Virginia University is 1 of only 11 research-intensive land-grant institutions offering a single health sciences campus with accredited Schools of Medicine, Dentistry, Nursing, and Pharmacy and a formative School of Public Health. WVU is West Virginia's major research and development center, and its only comprehensive doctoral-granting institution. Our faculty conduct research totaling over \$138 million in sponsored contracts and grants per year. The Carnegie Foundation for the Advancement of Teaching classifies WVU as a comprehensive doctoral institution with medical programs – placing it among only 50 such public and 28 private institutions nationwide.

Nominations, applications (including a cover letter, vitae, and list of 3 professional references), expressions of interest, requests for information, or confidential inquiries should be directed (preferably electronically) to:

David Siderovski, Ph.D., Chair, Search Committee
c/o Tammy S. Miller (tsmiller@hsc.wvu.edu)

Department of Microbiology, Immunology and Cell Biology
West Virginia University School of Medicine
Morgantown, WV 26506-9177

The position remains open until filled.

POSTDOCTORAL POSITION

BREAST CANCER TRANSLATIONAL RESEARCH

The Norma J. Vinger Center for Breast Care is seeking a talented postdoctoral fellow to work as a Research Scientist in conjunction with Dr. Simon Shelley conducting breast cancer research with Gundersen Medical Foundation in La Crosse, Wisconsin. The postdoctoral position will continue for up to four years. Experience with a broad range of molecular and cellular biology techniques is required with translation research directed towards breast cancer prevention, early cancer detection and diagnosis, and/or clinical molecular pathology. The successful candidate will be expected to contribute to undergraduate, graduate and professional teaching, and actively engage in community out-reach.

All candidates must have a Ph.D. and/or M.D. degree and evidence of first author papers published or accepted in peer-reviewed journals. The candidate will also have excellent written and verbal communication skills, and strong analytical capabilities.

Salary for the position will be commensurate with education and experience. Qualified applicants should apply on-line at gundersenhealth.org/research-fellow. Review of the applications will begin on May 1, 2014. The position will remain open until filled.

An overview of the Norma J. Vinger Center for Breast Care is available by visiting gundersenhealth.org/breast-care.

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- Cyber Security
- Machine Intelligence – Software Architecture as Autonomy Enabler

For more information and application procedure, please visit:

NTU – http://www3.ntu.edu.sg/trf/index_trf.html
NUS – <http://www.nus.edu.sg/dpr/funding/trf.html>
SUTD – <http://www.sutd.edu.sg/trf>

Closing date: 21 April 2014 (Monday)

Shortlisted candidates will be invited to Singapore to present their research plans, meet local researchers and identify potential collaborators in July / August 2014.



UNIVERSITY OF MIAMI
MILLER SCHOOL
of MEDICINE

Assistant Professor/Associate Professor/Professor Department of Cell Biology

The University of Miami Miller School of Medicine (UMMSM) is extending its ongoing investment in stem cell research and has openings for tenure-track faculty positions at all levels. The initiative is a collaborative enterprise at UMMSM and comprises the Department of Cell Biology, the Miami Project to Cure Paralysis (MPCP), the Sylvester Comprehensive Cancer Center (SCCC) and the Interdisciplinary Stem Cell Institute (ISCI).

Successful applicants will have demonstrated outstanding basic or translational/clinical research relevant to stem cell biology, transplantation and/or regenerative medicine. Areas of interest include the study of directed differentiation, reprogramming and morphogenesis involving adult somatic stem cells, embryonic stem cells, or induced pluripotent stem cells. Additional subjects of importance include basic or translational studies of cancer stem cells, preferably focusing on breast, or leukemia/lymphoma as well as other related malignancies. In addition, applications are sought from individuals whose research employs transplantation methodology or regenerative medicine centered on spinal cord injury or cardiology. Successful candidates, with a track record of funding, will have an appointment in the Department of Cell Biology, ISCI, and the appropriate Center/Institute reflecting the candidate's area of expertise.

Applicants must have a PhD and/or MD in Cell Biology, Neurology, Cancer Biology or other related field. Applicant must also be willing to teach graduate students and participate in university service activities and clinical service activities, if relevant. Interested individuals should submit a CV and short summary of research interests to:

Glen N. Barber, PhD
C/O Christina Villanueva
1550 NW 10th AVE
PAP BLDG – Room 510 (M-877)
Miami, FL 33136

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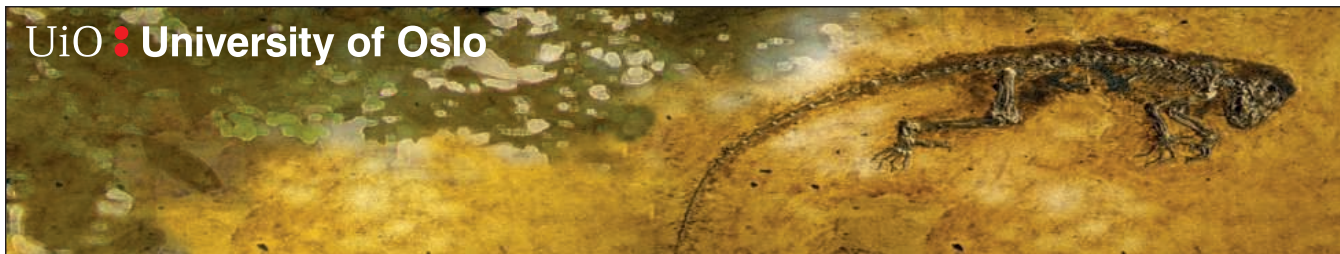
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The University of Oslo is Norway's largest institution of research and education with 28 000 students and 7000 employees. Its broad range of academic disciplines and internationally esteemed research communities make UiO an important contributor to society.

Systems Biology or Chemical Biology: Group Leader position with start-up package at the Biotechnology Centre of Oslo

Applications are invited for a group leader position (Ref. No.: 2014/1726) at the Biotechnology Centre of Oslo (BiO, www.biotek.uio.no), University of Oslo, Norway. BiO is a centre for molecular biology and biotechnology within the University of Oslo, which is co-localized with and closely coordinated with the Centre for Molecular Medicine Norway, Nordic EMBL Partnership (ncmm.uio.no).

The successful candidate will be offered a fixed-term, 5-year, renewable contract starting end 2014 or early 2015 with a budget of 1.8 mEUR over the first 5-year period. The group leader will be responsible for building up a strong research group and initiate a new independent research program either in systems biology or chemical biology. Qualification requirements include a PhD or equivalent degree, appropriate postdoctoral training, experience with supervision and a track-record of high-impact publications in top journals of biology or medicine. In addition, considerable weight will be put on the research plan proposed by the candidate and on how this will integrate and synergize with the activity in the region.

Application deadline **15th of March, 2014.**



For further information and a more comprehensive job description see:
<http://uio.easycruit.com/vacancy/1125923/70931?iso=no>

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**Department Head
Department of Biochemistry and Molecular Biology
Franklin College of Arts & Sciences**

The University of Georgia seeks an established scientist with creative vision to lead the Department of Biochemistry and Molecular Biology (<http://www.bmb.uga.edu>). With thirty-nine faculty members, the Department has internationally recognized research strengths in cancer research, stem cells, glycobiology, metalloenzymes, RNA biology, infectious disease, bioinformatics and structural biology (<http://www.bmb.uga.edu/research/departamental-research>), and robust undergraduate and graduate degree programs. We are looking for an individual with the vision to continue to develop the department within a collaborative and active life sciences research community.

Candidates should have a Ph.D. or equivalent in biochemistry or any relevant field, and be qualified for appointment as a full professor with tenure. In addition, candidates must have an outstanding record of academic accomplishments and funding, proven leadership and administrative skills, and a vision for future development of the Department. To apply, candidates should submit a cover letter summarizing their qualifications and vision for leadership, together with a complete curriculum vita that includes names and contact details of at least four individuals willing to provide letters of reference. Please provide this as a single PDF file to <https://www.franklin.uga.edu/jobs/>. Informal enquiries can be addressed to **Dr. Allen Moore**, head of the search committee (ajmoore@uga.edu). The committee will begin reviewing applications on **April 15, 2014**, and continue until the position has been filled. The start date for the position is August 2015 or negotiable.

The Franklin College of Arts and Sciences, its many units, and the University of Georgia are committed to increasing the diversity of its faculty and students, and sustaining a work and learning environment that is inclusive. Women, minorities and people with disabilities are encouraged to apply. The University is an EEO/AA Institution. Georgia is well known for its quality of life in regard to both outdoor and urban activities. The University of Georgia, the oldest state-chartered university in the United States, is a land/sea grant institution located in the city of Athens, 70 miles northeast of Atlanta. For more information on Athens see: <http://www.visitathensga.com>.



**Faculty Positions in Biomedical/Health Science Research
Institute of Biosciences and Technology, Texas A&M Health
Science Center, Houston, Texas**

The Texas A&M Health Science Center **Institute of Biosciences and Technology (IBT)**, <http://ibt.tamhsc.edu/>, is an internationally recognized leader in biomedical and health science research located at the Texas Medical Center in Houston, TX, with existing strengths in Epigenetics, Cancer/Stem Cell Biology, Infectious Disease, and Environmental Health.

The IBT is entering an expansion phase and will be recruiting multiple new faculty members into the existing Centers, including the new Center for Epigenetics & Disease Prevention (<http://www.ibt.tamhsc.edu/research/cedp/>). New hires will receive **highly competitive packages for salary, start-up, and support for graduate education, along with outstanding laboratory and office space** in the Texas A&M Health Science Center Alkek Building in the Texas Medical Center.

Applicants should have an M.D., Ph.D. or M.D./Ph.D. degree in biochemistry, cellular and molecular biology, microbiology, genetics or a related science, and an outstanding publication record; applicants at the Assistant Professor level should have at least 3 years post-doctoral experience. Current/ongoing extramural funding would be viewed favorably during the review process.

Successful candidates will be expected to establish an independent research group, conduct highly meritorious research, establish collaborations with other investigators in the Texas Medical Center and components in the Texas A&M University System, and to obtain significant extramural funding. Applications will be received and evaluated on a rolling basis, starting **April 3, 2014**. To apply, please send a cover letter, curriculum vitae, statement of research interests, copies of two key publications, and at least three reference letters to: **Drs. Roderick H. Dashwood and Magnus Höök, Search Committee Co-chairs, Institute of Biosciences and Technology, 2121 W. Holcombe Blvd., Houston, TX 77030-3303; E-mail: rdashwood@ibt.tamhsc.edu**.

The Texas A&M Health Science Center is an Affirmative Action, Equal Opportunity Employer.

THE UNIVERSITY OF HONG KONG



**Head and Professor
in the Department of Physics
(Ref.: 201400060)**

Applications are invited for appointment as Professor in the Department of Physics, preferably from July 2014 or as soon as possible thereafter. The appointee will also be appointed as Head of the Department of Physics for a three-year term starting from November 2015. Tenure may be offered to qualified candidates. Headship re-appointment will be subject to the University's established procedures.

Existing research activities in the Department include astrophysics and astronomy, condensed matter experiment, condensed matter theory, particle physics, semiconductor physics, and quantum information. Applicants should have a distinguished international reputation with a substantial research record. They should be able to provide leadership to enhance the Department's profile both locally and internationally as a centre of excellence in research and teaching. Experience in management would be an advantage.

A globally competitive salary commensurate with qualifications and experience will be offered. At current rates, salaries tax does not exceed 15% of gross income. The appointment will attract a contract-end gratuity and University contribution to a retirement benefits scheme, totalling up to 15% of basic salary, as well as annual leave, and medical benefits. Housing benefits will be provided as applicable.

For enquiries about the existing research activities and the specific job requirements, please write to Professor Sun Kwok, Dean of Faculty of Science (e-mail: sunkwok@hku.hk). Interested applicants should submit a completed application form together with an up-to-date C.V., a detailed publication list, a research plan, and a statement on teaching philosophy to scphy@hku.hk. Please indicate clearly "Ref.: 201400060 (Head and Professor in the Department of Physics)" in the subject of the e-mail.

Application forms (341/1111) can be obtained at <http://www.hku.hk/apptunit/form-ext.doc>. Further particulars can be obtained at <http://jobs.hku.hk/>. **Closes April 30, 2014**. The University thanks applicants for their interest, but advises that only shortlisted applicants will be notified of the application result.

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**Department of Cellular and Molecular Physiology
The Pennsylvania State University College of Medicine
New Faculty Positions**

The Department of Cellular and Molecular Physiology in the Pennsylvania State University College of Medicine invites applications from outstanding Ph.D. and/or M.D. scientists for multiple Tenure/Tenure-Track positions at the rank of Assistant or Associate Professor. The Department is undergoing a major expansion of research within the thriving new research environment created in the Milton S. Hershey Medical Center, located in Hershey, Pennsylvania.

Under leadership of the new Chair, Dr. Donald Gill, the Department is building in the broad areas of cellular, molecular and integrative approaches toward translational research in physiology. The search is open to applicants in many areas of physiological research including those with translational approaches to studying signaling mechanisms, channel structure/function and channel-related diseases, mitochondrial dysfunction and oxidative stress, cardiovascular and musculoskeletal diseases, and metabolic disorders. We are seeking early stage or established investigators with strong records of research accomplishment who will complement and expand these departmental research interests. The Department enjoys strong interactions with the Heart and Vascular Institute, the Institute for Personalized Medicine, and the NIH-supported Clinical and Translational Science Institute within the Penn State Hershey Medical Center. Priority will be given to those applicants who have established funded research programs or have recently obtained funding to transition into independent research. Competitive start-up packages, recently renovated laboratory space, and strong core facilities, together provide an outstanding research environment within the Department.

Interested applicants should submit a cover letter, curriculum vitae, and statement of research plans (merged within a single pdf) to: **C&MPhysioSearch@hmc.psu.edu**. Further information about the Department and the successful and appealing Hershey environment are provided at the Department website: www.med.psu.edu/physiology and at: <https://wikispaces.psu.edu/x/VAJ4Cw>.

Penn State is committed to affirmative action, equal opportunity and the diversity of its workforce. Minorities and women are encouraged to apply.

POSITIONS OPEN

FACULTY POSITION in Infectious Disease Research University of Nebraska Medical Center

ASSOCIATE or FULL PROFESSOR focused on pathogenesis of bacterial infection, with special emphasis on virulence factors required for infection. Priority for investigators interested in Gram-positive pathogens and/or select agents, and experience in vaccine development. Successful candidate will have a track record of extramural funding, publications and original concepts. Appointee will possess a strong commitment to collaborative approaches to research and will participate in the academic missions of the department, including graduate teaching in host-pathogen interactions and mentoring of students and postdoctoral fellows. Resources include collaborators and programs within the NIH-funded Center for Staphylococcal Research (website: <http://www.unmc.edu/pathology/csr/>). Applicants must have a Ph.D., M.D., or equivalent degree, and research experience in bacterial genetics and pathogenesis. Applications are being accepted online at website: <http://jobs.unmc.edu/postings/18433>. Please include a letter of interest, curriculum vitae, description of future research plans, and the names of three references. Individuals from diverse backgrounds are encouraged to apply.

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